



**Survival of waterborne enteric viruses in the Msunduzi River in
Pietermaritzburg**

Submitted in the fulfilment of the requirements of the degree of Masters
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Justine Olivia Moodley

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Supervisor: Prof. F.M. Swalaha

Co-Supervisor: Prof. S.K. Kuttan-Pillai

Abstract

Waterborne enteric viruses are ubiquitous in a riverine environment. However, there has been no direct correlation between bacterial faecal indicators and viral contamination in water. Therefore, a viral indicator or combination of bacterial and viral indicators may be necessary to determine contamination in environmental waters and their sources. Enteric viruses are detected in water using various methods including qPCR, RT-PCR, cell culture and sequencing. In this study, adenovirus, norovirus GI and GII, rotavirus A and hepatitis A were detected in all samples collected from the Msunduzi River in Pietermaritzburg using a digital PCR method. Results obtained indicated that environmental factors, including ammonia, temperature, pH, phosphates, rainfall, suspended solids, turbidity and TOC, were found to affect enteric virus survival in a riverine environment. It was further observed that anthropogenic activities such as the Dusi Canoe Marathon impacted the distribution of enteric viruses throughout the Msunduzi River. Adenovirus 40 and 41 and all serotypes were detected together with other enteric viruses and bacterial indicators proving to be a possible indicator for viral pathogens. Little to no correlation was found between enteric viral pathogens and enteric microbial loads in this river. Future work should include the investigation of other possible viral indicators and forming an indicator complex that includes both bacterial and viral indicators for water quality based microbial risk assessment framework.

Approval

I, the undersigned, hereby approve the final submission of the following dissertation at the Durban University of Technology.

Prof. F.M. Swalaha

Prof. S.K. Kuttan-Pillai

Declaration by student

Survival of waterborne enteric viruses in the Msunduzi River in Pietermaritzburg

Justine Olivia Moodley

I, Justine Olivia Moodley, hereby declare that this dissertation and the content herein is entirely my own work. It has never before been submitted for any degree to any other university.

Reference declaration

I, Justine Olivia Moodley, Prof. F.M. Swalaha and Prof. S.K. Kuttan-Pillai, do hereby declare that in respect to the following dissertation: Survival of waterborne enteric viruses in the Msunduzi River in Pietermaritzburg, as far as we know and can as certain, no other similar dissertation exists, all references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

Signature of Student

Dedication

To my family, for their never ending support and prayers.

“People with their minds set on you, you keep completely whole, steady on their feet, because they keep at it and don’t quit. Depend on God and keep at it because in the Lord God you have a sure thing.” Isaiah 26:3-4

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List of abbreviations

AdV – adenovirus

BOD – Biochemical Oxygen Demand

COD – Chemical Oxygen Demand

DO – Dissolved Oxygen

dsDNA – double-stranded deoxyribonucleic acid

dsRNA – double-stranded ribonucleic acid

FIB – faecal indicator bacteria

HAV – hepatitis A virus

NoV GI – norovirus genogroup I

Nov GII – norovirus genogroup II

NoV- norovirus

PEG – Polyethylene Glycol

RVA – rotavirus A

SRP – Soluble Reactive Phosphate

ssDNA – single-stranded deoxyribonucleic acid

ssRNA – single-stranded ribonucleic acid

TOC – Total Organic Carbon

CHAPTER ONE – INTRODUCTION

1.1 Context of research

In dry and semi-arid areas, there is a correlation between rising human population levels, insufficient rainfall and a shortage of drinkable water sources. Globally, millions of people do not have access to microbiologically safe water that can be utilised for recreational, household and other uses (Gibson *et al.*, 2011).

Waterborne virus outbreaks represent a significant public health concern and are related to the use of poorly treated water. Approximately 200 different kinds of human disease-causing enteric viruses are found in sewage-contaminated aquatic environments. They represent the largest amount of different species of enteric pathogens in water (Clasen *et al.*, 2014; Gerba and Betancourt, 2017). The major waterborne human pathogenic viruses include norovirus (Nov), adenovirus (AdV), hepatitis A (HAV) as well as rotavirus (RV). The faeces of infected individuals contain high concentrations of human enteric viruses resulting in high viral particle numbers in raw sewage (Gerba *et al.*, 2018). Waterborne diseases have continued to kill more than 2 million people each year. In 2022, viral hepatitis caused approximately 1.3 million deaths (WHO, 2024).

In the environment, viruses have been found to survive longer than bacteria (Alhamlan *et al.*, 2015). Human pathogenic viruses are more resistant to removal during wastewater treatment than bacterial indicators and can remain viable after disinfection (Ruhanya, 2016; Gerba *et al.*, 2013). Accumulating evidence regarding waterborne disease outbreaks has revealed that the absence of bacterial indicators does not necessarily decrease the likelihood of other pathogens that can cause disease outbreaks (Zhu *et al.*, 2018; Forlenza *et al.*, 2012).

Due to the historically high cost of detection techniques, human enteric viruses have not been routinely monitored in the water industry. Faecal bacteria are instead used as indicators by health organisations and environmental agencies to monitor the quality of water. These bacteria are detected using culture-based assays, particularly total coliforms and *E. coli*

(Ferguson *et al.*, 2013). Currently, there are no standard methods for monitoring viruses in potable water and wastewater as it is not a requirement included in the South African National Standard (SANS) guidelines for water monitoring. Detecting waterborne pathogens is a tedious, costly and complex procedure due to pathogen types and variability as well as the complex techniques required for the concentration and detection of viruses. There are generally low numbers of viruses in water, making their detection even more complex (Gibson, 2014; Bofill-Mas and Rusiñol, 2020; Li *et al.*, 2023).

Molecular techniques have allowed for more rapid detection of viruses, especially those that cannot be grown in cell culture, as they have better sensitivity (Dubrou *et al.*, 1991; Mattison and Bidawid, 2009). The monitoring of viral pathogens should ideally be included in the classification of microbiological hazards in water in the future due to the higher chance that they would be identified in various aquatic ecosystems. By doing this, it will be possible to address the associated health risks and develop effective prevention schemes (Rose and Molloy, 2007).

Surface waters are more susceptible to contamination because they are open bodies of water. Rivers are the most pertinent transporters of pathogens. They are the receiving water for treated wastewater which, despite treatment, may still comprise high volumes of water with potentially viable viruses at varying concentrations (Corpuz *et al.*, 2020). Therefore, pathogens can be introduced into surface waters through inadequately treated wastewater effluent, broken and blocked or overflowing sewers, especially below any hospital sewer outfall, agricultural land runoff, informal settlements and irrigation waters. In addition, rivers are subject to varying seasonal impacts, which can significantly impact pathogen concentrations. Droughts reduce the volume and flow velocity of rivers. This increases the local concentration of pathogens in contaminated waters due to a reduced dilution rate by receiving streams. Heavy rainfall can lead to a shift in the direction of the flow of water and localised flooding events (Cann *et al.*, 2013) and despite the higher dilution, also results in faster transport and reduced pathogen removal time before reaching potentially vulnerable downstream

communities and/or users. Numerous human viral pathogens are detected yearly or seasonally in surface waters that receive wastewater (Seidel *et al.*, 2016). Dumping associated with informal settlements is also causing concern where there is no control over waste being dumped into rivers and coastal environments (Rodríguez-Díaz *et al.*, 2009).

Waterborne enteric viruses can cause potential harm to those using river water for domestic use, be it bathing or washing clothing. People may unintentionally become exposed to waterborne infections by using river water for irrigation and other agricultural uses. Viruses are known to be more resistant to disinfection methods than enteric indicator bacteria such as *E. coli* (Lanrewaju *et al.*, 2022; Worley-Morse *et al.*, 2019; Tree *et al.*, 2003; Korajkic *et al.*, 2022). Survival of viruses against disinfection is due to numerous factors such as the mechanical stability or plasticity of the viral capsid, the level of secondary structure present and the level of interaction and protection by viral capsids (Prevost *et al.*, 2016). Monitoring of virus prevalence both pre- and post-disinfection would therefore provide a better indication of further steps to be taken to ensure the removal of virus particles from wastewater. Monitoring virus prevalence would indicate the effect of different waterborne pathogen sources on the viral load in surface water.

Viruses can be separated into two categories: enveloped viruses with a lipid membrane and non-enveloped viruses. Due to the instability of the envelope, enveloped viruses are more susceptible to changes in temperature and pH. Other abiotic factors that could have a possible impact on virus survival in river water include rainfall, turbidity, biochemical oxygen demand (BOD) and total organic carbon (TOC).

1.2 Scope of study

The distribution of enteric waterborne pathogenic viruses in/of major watercourses in Kwa-Zulu Natal is currently unknown. Hence, the Msunduzi River in Pietermaritzburg was chosen as the area of study as it is used for both domestic and recreational purposes such as the Dusi Canoe Marathon held annually. Previous studies on this watercourse have focused primarily

on bacterial indicators to determine water quality and have formed the basis of epidemiological studies carried out after the canoe race. The South African National Standard (SANS) is currently under review to include the detection of enteric viruses in water as sole reliance can no longer be placed on bacteria as indicators of potential pathogens in water. The study entailed the detection of the most common waterborne pathogenic enteric viruses, which may be present in surface waters and how their presence could potentially affect public health. This was accomplished using a detection method adapted from Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR Part 1: Method for quantification, ISO 15216-1, together with digital PCR. Samples were taken fortnightly for 6 months at 6 sampling sites upstream in the river to include seasonal variation.. Wastewater was not the focus of this study, and much research has been done in this field and enteric viruses are known to be present, hence no samples were analysed downstream or from wastewater plants.

1.2.1 Problem statement

Enteric viruses are not routinely monitored in a riverine environment or their impact associated with anthropogenic activities assessed due to costly and laborious detection methods.

1.2.2 Hypothesis

It is hypothesised that enteric virus levels in the river would be low upstream based on bacterial indicator levels. However, human activities and environmental factors would impact these concentrations, particularly the canoe race and seasonal changes.

1.2.3 Aim

To assess the distribution of waterborne pathogenic viruses in a riverine environment and determine the effects of anthropogenic and abiotic factors on their survival and distribution.

1.2.4 Objectives

- i. To adapt and validate a method to detect pathogenic viruses in river water using digital PCR.
- ii. To determine the effects of various human activities on the presence and survival of viruses in river water.
- iii. To correlate viral loads with abiotic factors in order to predict the survival ability of viruses during differing environmental conditions.
- iv. To predict and establish the relationships among the viruses detected in the water with enteric microbial indicator loads using statistical correlation techniques.

CHAPTER TWO – LITERATURE REVIEW

2.1 Introduction

Water is essential to life and requires efficient monitoring to protect it. Every country has its own water bodies, water treatment processes, monitoring systems and water policies in place. Water is a vulnerable resource due to pollution and excessive exploitation although it is recyclable. In addition, it is an important source of human exposure to gastro-enteric disease due to the consumption of faecal contaminated water or food that is irrigated with it (Fuster *et al.*, 2016). The crucial principles behind water treatment processes are to avoid contamination of water sources and protect public health against the spread of pathogenic organisms (Osuolale and Okoh, 2017).

Escherichia coli, a faecal coliform, is the most common bacterial indicator for the presence of faecal pollution in all forms of water as it is essentially ubiquitous in the mammalian gut. Traditionally, faecal indicator bacteria (FIB) are utilised to determine the presence of faecal contamination by both humans and animals although, they cannot differentiate between the two sources. Another limitation when using bacterial indicators is the lack of a relationship between the indicator and the occurrence of human pathogens contained in environmental samples (Payment and Locas, 2011). Other indicators used to test the quality of water are human enteric viruses which can also be used as indicators of faecal pollution. In recent years, viruses have become the most prominent cause of waterborne gastroenteritis in humans of all ages. There are over 100 types of human enteric viruses that are present in sewage (Dumontet *et al.*, 2001). Viruses are shed in the faeces of infected persons and their numbers range between 10^5 and 10^{11} viruses per gram. This indicates the increased likelihood of contamination up to surface water and groundwater levels through overland flow and soil infiltration respectively (Corsi *et al.*, 2014). Implementing the routine monitoring of viruses can be an impractical affair due to the high cost of sampling and analysis to produce results promptly that will add value, but also due to problems that will be encountered when trying to detect a full range of viruses that may occur.

Environmental emissions such as effluents from sewage treatment plants can affect human health. Between 15 to 20% of community diarrheal disease has been due to unsafe drinking water whereas higher percentages are associated with waterborne diarrheal disease (Batabyal *et al.*, 2016). The impact of the environment on human health can be assessed through different environmental assessment methods. Another common approach is quantitative risk assessment (QRA) which can be utilised to determine the health risk to the surrounding community after exposure to pathogens and harmful chemicals (Kobayashi *et al.*, 2015). Other impacts need to be considered when performing environmental impact assessments including ecosystem quality, resource depletion and occupational safety for water treatment plants (WTP) to improve the assessment of environmental impacts on public health (Kobayashi *et al.*, 2015).

A significant fraction of freshwater ecosystems is made up of riverine environments. These riverine environments are exposed to the combined harmful impacts of anthropogenic stresses which are caused by numerous factors such as habitat degradation and invasive species. There is an increased demand for water and human water security as a whole (Todd *et al.*, 2017). Climate change influences the quality of water in drinking water sources namely rivers, lakes and marine waters. These environmental waters are also used for recreational activities. Changes that pose a risk to water supplies include changes in rainfall patterns, increased temperatures and natural weather-related disasters. With urban expansion on the rise, it has contributed to elevated microbial loads which in due course are discharged into receiving waters.

Surface waters are open bodies of water making them more susceptible to contamination. Rivers are the most prevalent transporters of pathogens as they are the receiving waters for treated wastewater as well as other potential sources of human and animal pathogens (Seidel *et al.*, 2016). River networks play a crucial role as epidemiological corridors. Waterborne disease infections are caused by ingestion or contact with pathogenic organisms present in water. Contaminated river waters represent a public health risk where the burden of

waterborne infections is due to unsafe water supplies which directly and indirectly affects exposure and transmission rates (Rinaldo *et al.*, 2018).

Human enteric viruses that are present in a riverine environment could potentially reach estuaries. Their fate is controlled by environmental conditions as well as their capability to decay over a period. It is important to understand the conditions under which enteric viruses are retained in the estuary and transported thereafter. This could radically improve the water quality management approach and simultaneously reduce the health risks posed to the public (Robins *et al.*, 2019).

2.2 Types of waterborne enteric viruses

Enteric viruses are commonly connected with waterborne disease outbreaks (Farkas *et al.*, 2018). Viruses have various mechanisms of action and with a virion ranging between 10-100 nm in diameter. The virion contains the DNA or RNA genome and is encapsulated in a protein coat known as a capsid. The information needed to seize control of a host cell and initiate viral replication is transferred by the viral nucleic acid. It involves the storage and expression of genetic information for the synthesis of all proteins. The capsid protects the nucleic acid from nucleases and facilitates virus attachment to the host cell. Therefore, a host organism is required for viruses to reproduce (Altintas *et al.*, 2015). Viruses are unable to replicate outside of the host cell and are considered intracellular parasites. Once the host cell is infected, the infected organism excretes large amounts of virus progeny, thereby posing a threat to surrounding healthy cells. Following infection, the phage distributes its genetic material into the bacterial host cell, allowing it to replicate via either the lytic or lysogenic pathways (Makky *et al.*, 2021). Some viruses are resistant to high temperatures, such as adenovirus, poliovirus and polyomavirus, acidic conditions, pressure as well as disinfectants (Sauerbrei and Wutzler, 2009; Belon and Frick, 2011). Various DNA viruses can alter DNA repair mechanisms to improve viral DNA replication (Erickson and Garcea, 2019). Human enteric viruses are not unusual contaminants that are found in rivers worldwide. The most common human enteric viruses are listed in Table 1.

Table 1. Characteristics of the most common human enteric viruses

| Virus | Family | Virion diameter (nm) | Genome type | Clinical illnesses | Sources | Route of transmission | Reference |
|-----------------------------|-----------------------|----------------------|-------------|---|--|---|----------------------------------|
| <i>Hepatitis A</i> | <i>Picornaviridae</i> | 27 – 32 | +ssRNA | Hepatitis, liver disease | Contaminated food and water | Faecal-oral route | (Levican <i>et al.</i> , 2023) |
| <i>Hepatitis E</i> | <i>Hepeviridae</i> | 27 – 34 | +ssRNA | Hepatitis | Humans, pigs and wild boar, contaminated food and drinking water | Faecal-oral route | (Liu <i>et al.</i> , 2023) |
| <i>Adenovirus 40 and 41</i> | <i>Adenoviridae</i> | 60 – 100 | dsDNA | Gastroenteritis, conjunctivitis, pharyngitis, pneumonia, respiratory disease, urinary infection | Wastewater, surface water, recreational water, disinfected potable water | Faecal-oral and respiratory | (Shieh, 2021) |
| <i>Norovirus GI and GII</i> | <i>Caliciviridae</i> | 26 – 35 | +ssRNA | Gastroenteritis, conjunctivitis, pharyngitis | Contaminated water and food | Person-to-person, foodborne, waterborne | (Chhabra <i>et al.</i> , 2019) |
| <i>Enterovirus</i> | <i>Picornaviridae</i> | 15 – 30 | +ssRNA | Meningitis, gastroenteritis, conjunctivitis, pharyngitis, encephalitis, myocarditis | Contaminated water, respiratory secretions | Faecal-oral route | (Yoo <i>et al.</i> , 2023) |
| <i>Rotavirus A</i> | <i>Reoviridae</i> | 70 | dsRNA | Gastroenteritis | Contaminated surfaces, contaminated water | Faecal-oral route | (Crawford <i>et al.</i> , 2017) |
| <i>Sapovirus</i> | <i>Caliciviridae</i> | 27 – 40 | +ssRNA | Gastroenteritis | Contaminated water, food and surfaces | Faecal-oral route | (Oka <i>et al.</i> , 2015) |
| <i>Astrovirus</i> | <i>Astroviridae</i> | 28 – 30 | +ssRNA | Gastroenteritis | Contaminated food and water | Faecal-oral route | (Moser and Schultz-Cherry, 2008) |
| <i>Coronavirus</i> | <i>Coronaviridae</i> | 125 | +ssRNA | Respiratory tract infection, gastroenteritis, pneumonia, bronchitis | Respiratory secretions | Person-to-person contact | (Smith, 2021) |
| <i>Aichivirus</i> | <i>Picornaviridae</i> | 30 | +ssRNA | Gastroenteritis | Shellfish | Faecal-oral route | (Rivadulla and Romalde, 2020) |
| <i>Bocavirus</i> | <i>Parvoviridae</i> | 18 – 26 | ssDNA | Respiratory tract infection, gastroenteritis | Respiratory secretions | Animal contact | (Guido <i>et al.</i> , 2016) |

Environmental waters contaminated by human pathogens, namely enteric viruses, are identified as a potential threat to human health, especially those using environmental waters for recreational purposes. Human enteric viruses are extremely stable in the environment and can persist for long periods in the water. This is because the majority lack a lipid envelope (Prez *et al.*, 2015). Enteric viruses are considered to be emerging waterborne pathogens. This

is due to their molecular structures that allows them to be more resistant to removal or inactivation in conventional water treatment techniques. The persistence of enteric viruses in water can increase with virus adsorption to particulate matter present in the water (Bosch *et al.*, 2007).

Adenovirus (AdV) group F, enterovirus (EV) group A-D, hepatitis A and E viruses (HAV and HEV), norovirus genotype I and II (NoVGI and NoVGII) and rotavirus (RV) A are viruses commonly associated with waterborne diseases (Ashbolt, 2015; Perkins and Trimmier, 2017; Chen *et al.*, 2021).

2.2.1 Adenovirus

Adenoviruses also form part of the United States Environmental Protection Agency (USEPA) list of drinking water contaminants. Human adenoviruses (HAdV) are non-enveloped double-stranded DNA viruses with a genome size of approximately 26 to 45 kb, consisting of an icosahedral capsid of between 60 to 100 nm and are found within the *Adenoviridae* family. There are more than 68 different types that have been divided into seven groups, namely A to G (Girardi *et al.*, 2019; Dias *et al.*, 2018; Kajon *et al.*, 2019). At 4°C and 20°C, human adenovirus serotype 41 can stay stable and infectious for over 70 days, and it is most abundant in surface waters and sewage. It is recognised as the second most common cause of paediatric gastrointestinal infections globally (Leifels *et al.*, 2016). Adenovirus infections are usually asymptomatic as compared to HAV and HEV, which cause acute gastroenteritis. Therefore, they are difficult to diagnose due to infected persons displaying little or no symptoms of infection (Altintas *et al.*, 2015).

Adenovirus (AdV) is host-specific and can potentially be a viral Microbial Source Tracking (MST) indicator for faecal contamination of human origin. It is known to persist in domestic wastewater (Symonds *et al.*, 2017). Some types of AdV have shown enhanced resistance to disinfection methods, namely UV light and chlorination treatment because of the outer capsid proteins' robust structure and their dsDNA. Viral genomes and/or exterior structures can be

destroyed by disinfectants, which is one of the processes by which they inactivate viruses. Nucleic acids are destroyed by UV exposure. Furthermore, chlorine dioxide reportedly damages viral proteins as well as nucleic acids (Imai *et al.*, 2022). Adenoviruses also exhibit enhanced stability under adverse conditions such as changes in pH and temperature (Fong and Lipp, 2005). Adenovirus has been found to survive for longer periods in water compared to other viruses which may be due to their double stranded DNA (Gerba, 2015).

2.2.2 Norovirus

Norovirus (NoV), previously known as Norwalk virus, belongs to the *Caliciviridae* family and contains positive-sense single-stranded RNA approximately 7.5 kb in length. It is the predominant cause of acute non-bacterial gastroenteritis (Seo *et al.*, 2014). Norovirus comprises of five genogroups (GI-GV), in particular, GI and GII are recognised as the main cause of viral gastroenteritis with common symptoms such as vomiting and diarrhoea. It is the most noteworthy etiological agent in recreational waterborne outbreaks that are documented followed closely by adenoviruses. Waterborne outbreaks due to NoV originating from contaminated potable water and recreational waters have been reported (Kishida *et al.*, 2012). The most common transmission pathway is person-to-person contact, however, it can be spread via various other pathways such as contaminated food, shellfish and water. With a probability of infection of approximately 49% for a single viral particle, noroviruses are highly contagious (Teunis *et al.*, 2008; Rusiñol *et al.*, 2015). Airborne transmission has also been reported (Greening, 2006).

Elevated concentrations of NoV can be introduced to the environment via the discharge of treated or untreated sewage. This virus has shown resistance to inactivation and has been found to have similar numbers in both influents and effluents from wastewater treatment plants (Hewitt *et al.*, 2011). Environmental waters impacted by different community sizes were observed to characterise NoV diversity and seasonality. It was found that one or two genotypes were present in smaller communities as compared to larger ones where more than

six different genotypes were found. Norovirus is more prevalent during winter (Fernández *et al.*, 2012).

2.2.3 Rotavirus

Rotavirus (RV) belongs to the family *Reoviridae* and is listed as a relevant waterborne pathogen by the World Health Organisation. It is a double-stranded RNA virus that is categorised into eight serogroups, namely A to H and has a genome size of approximately 18.5 kb (Desselberger, 2014). Human gastroenteritis is caused by serogroups A, B, and C, whereas group A is known for causing severe diarrhoea in young children. In addition to contaminated food and water, rotavirus can spread from person to person (Seo *et al.*, 2014). In surface waters, it has been detected more often than norovirus. Although the majority of cases have been documented all year round globally, it is thought of as a winter virus because it is more frequently found during the winter months (Shamsollahi *et al.*, 2019; Osuolale and Okoh, 2017; Okaali *et al.*, 2021).

2.2.4 Hepatitis A

The *Picornaviridae* family includes hepatovirus, enterovirus and rhinovirus. The hepatitis A virus (HAV) is the sole member of the genus hepatovirus. It is one of the more well-characterised human viral pathogens in water (Fuster *et al.*, 2016). Viral replication occurs in the liver resulting in hepatic injury. With a genomic size of 7.5 kb, the non-enveloped, single-stranded RNA HAV virus particles are more stable in the environment than the other picornaviruses (Chou and Williams-Hill, 2018). They have shown resistance to temperatures up to 60°C, disinfectants and a low pH, which enables their transmission (Ragab *et al.*, 2024). There are six genotypes of HAV where genotypes I-III are divided into further subgenotypes A and B. Transmission occurs via the faecal-oral route, person-to-person contact or consumption of food and water which is faecally contaminated (Takuissu *et al.*, 2023).

An estimated 1.4 million HAV cases occur annually around the world (O'Neil, 2018). When infected with HAV, a number of clinical symptoms manifest, such as fever, vomiting, liver

enlargement, cholestatic jaundice, anorexia, and abdominal pain. As few as 10-100 viral particles that are ingested can cause infection (Mandli *et al.*, 2017). The incubation period of acute HAV can be anywhere between 15 to 50 days. Jaundice is the most noticeable symptom, and the frequency of symptoms is closely correlated with the age of the infected individual. People who exhibit symptoms are thought to be the most contagious during the 14 days when the virus is most prevalent in the patient's faeces. Of children under the age of six, over 70% do not exhibit any symptoms. They shed HAV for almost 10 weeks longer than adults; hence they are at a high risk for spreading infection (Muñoz-Martínez *et al.*, 2018; O'Neil, 2018). Hepatitis A virus exhibits a major health risk that causes significant morbidity as well as economic loss. Economic progress has a strong correlation with the prevalence of HAV. The prevalence of HAV infection has declined in underdeveloped nations. The main cause of this is improved availability to potable water (Manzano *et al.*, 2018; Koroglu *et al.*, 2017). However, the infection is known to confer lifelong immunity where no chronic infection has been reported (Yu *et al.*, 2015).

2.2.5 Hepatitis E

The *Hepeviridae* family includes the Hepatitis E virus (HEV), which has four human-affecting genotypes. Although it causes acute hepatitis, it can also cause chronic hepatitis in certain circumstances. In low-income nations, waterborne epidemics have been linked to genotypes 1 and 2, which are known to infect only humans (Randazzo *et al.*, 2018). This single-stranded positive-sense RNA virus is non-enveloped, with a diameter of 27–34 nm and a size of approximately 7.2 kb. It causes acute hepatitis through faecally contaminated water (Seo *et al.*, 2014). Numerous HEV epidemics linked to drinking water polluted by faeces have occurred in underdeveloped nations (Givens *et al.*, 2016; Tarantino *et al.*, 2016). Until symptoms appear, infected individuals defecate up to 10^{11} genome copies per gram of faeces. Thus, direct contact with infected individuals or ingestion of contaminated food or water results in transmission through the faecal-oral pathway. In endemic areas, contaminated waters serve as the main vector for HEV transmission (Randazzo *et al.*, 2018). An HEV infection typically

takes 15 to 60 days to incubate (Ishida *et al.*, 2018). Numerous factors, including living conditions, behavioral patterns, and host immunological status, interact to influence the occurrence of HEV within a given community. In addition to viral characteristics that influence genetic diversity and the virus's host range, environmental factors also influence HEV's persistence in various locations. Environmental samples, including soil and wastewater effluents, have been confirmed to contain the hepatitis E virus (Sooryanarain and Meng, 2019).

2.2.6 Enterovirus

Enterovirus (EV) belongs to the *Picornaviridae* family. It is a spherical, non-enveloped virus made up of positive-sense single-stranded RNA. Its capsid is between 15 and 30 nm in diameter. Its genome size is approximately 7.2 to 8.5 kb (Chien *et al.*, 2019). EV-caused infections in humans can be asymptomatic and are known to rise in the summer, when more people bathe and engage in other recreational water activities. Enterovirus has been used as a marker of infectious illnesses in environmental waters and is known to proliferate in cell culture (Girardi *et al.*, 2019; Prez *et al.*, 2015).

2.3 Riverine water quality

Water is one of the main channels via which humans are exposed to microbial pathogens. Freshwater recreational water environments can be described as any estuarine or freshwater area where recreational usage may occur by a substantial amount of users (WHO, 2013). The major concern is placed on the uses of water for domestic or recreational purposes where water contact or the risk of water ingestion may be involved. There are a fair number of hazards and risks associated with the recreational use of freshwater such as injury or illness and possible exposure to free-living pathogenic micro-organisms. These hazards and risks can result in infection caused by ingestion, inhalation or contact with pathogenic bacteria, fungi, viruses and parasites present in the water due to faecal contamination and can be

carried by recreational users and animals or can be naturally present (Pakasi, 2018; Andreeva, 2021).

There are various pathways through which pathogens are introduced to surface waters, in addition to home and industrial waste disposal, such as stormwater, industrial effluents, sewage and animal manure runoff. The occurrence of waterborne outbreaks of enteric viruses is well documented (Guerrero-Latorre *et al.*, 2011; Maunula *et al.*, 2009; Sekwadi *et al.*, 2018; Carol *et al.*, 2021; O'Reilly *et al.*, 2007; Hewitt *et al.*, 2007). There have been many investigations related to recreational activities, such as swimming, canoeing and fishing, in virus-contaminated waters with gastrointestinal disease (Sunger and Haas, 2015; Natarajan and Miller, 2016; McGinnis *et al.*, 2022; Dale *et al.*, 2010). However, only a small portion of outbreaks are ever reported or researched. Outbreaks of waterborne microbial disease have been directly related to the faecal pollution of urban estuaries. Limited investigations have been carried out to determine the levels of reference pathogens within the system (Henry *et al.*, 2016).

More adequate surveillance and management strategies are required to evaluate the risk of waterborne pathogen transmission (Poma *et al.*, 2012). There are many variables to consider when designing a routine monitoring scheme or rivers such as sampling points, frequency of sampling, the volume of samples to be taken, methods to be adopted and climatic conditions. River water quality characteristics are subject to change, therefore screening indicators should constantly be updated. One study suggested an index analysis be carried out over a year to determine the frequency of monitoring for screening indicators for a more accurate representation of the quality of a particular body of water (Liu *et al.*, 2012). River flow and other hydrological factors cause difficulties when analysing the changes in river water quality. The frequency of sampling is a crucial component to take into account while creating a network for monitoring water quality (Liu *et al.*, 2014). In order to integrate both online water quality monitoring and early warning detection systems, a recent study recommended that future research entail the continuous collection of water samples throughout the year, as well as

additional water samples and water quality parameters of inland waters in various environments (Cao *et al.*, 2022). Diverse monitoring purposes cannot be satisfied by a single approach or set of procedures. The majority of monitoring strategies used today focus on effect detection or targeted exposure (Altenburger *et al.*, 2019).

Despite the large quantities of human pathogens being released into rivers through sewer network problems, wastewater treatment plant effluents, contaminated surface runoff (land deposition of faeces or inadequate pit latrines) or direct contamination, surface water is widely used for recreational and irrigation purposes as well as a source for drinking water. There has been an increasing number of studies being done on enteric viruses in potable water, partly due to the outbreaks of gastroenteritis associated with the ingestion of contaminated food and water, as well as improved detection methods for viruses (Sorensen *et al.*, 2021; Özgüler *et al.*, 2022; Zhang *et al.*, 2018; Zhou *et al.*, 2019). A study suggested that enteric viruses are preserved and remain infectious for long periods in the environment (Prevost *et al.*, 2016). The long-term survival of poliovirus 1, echovirus 7 and coxsackievirus B3 was assessed at temperatures: -20, 1 and 22°C. The average viral inactivation was found to be 6.5 to 7 log₁₀ over 8 weeks at 22°C, 4 to 5 log₁₀ over 12 weeks at 1°C and 0.4 to 0.8 log₁₀ over 12 weeks at -20°C (Hurst *et al.*, 1989). Rotaviruses can also persist in freshwater for several days. A 99% reduction of rotavirus was found in seeded river water over a period of 10 days at 20°C. However, at 4°C, a 99% reduction took 32 days (Raphael *et al.*, 1985). Hepatitis A showed little to no decay over a period of 48 days in river water (Springthorpe *et al.*, 1993). Waterborne viruses have therefore become a research priority for polluted waters. Public waters contaminated by waterborne viruses are a global health concern and viruses being transmitted via ingestion of or contact with polluted waters is well known (Fout *et al.*, 2012). This was found to be evident with the detection of the recent SARS CoV2 genetic material in surface waters (Guerrero-Latorre *et al.*, 2020; Haramoto *et al.*, 2020; Rimoldi *et al.*, 2020). Numerous enteric viruses have been quantitatively determined in riverine environments using conventional and quantitative real-time PCR techniques, such as adenovirus, enterovirus,

rotavirus and norovirus. However, if monitoring is limited to a few virus types, it could provide a misleading image of the risk that the water being tested poses if other pathogens are present as well. (Kistemann *et al.*, 2016).

The river basin area can be regarded as interdependent and interconnected elements and components that can affect water levels or impact other areas, including social or economic conditions. River basin management can be used to reduce the effects of climate change to safeguard water resources. This can be achieved by using specific markers to identify faecal contamination sources in environmental waters (Rusiñol *et al.*, 2014). A changing climate and the increase in intensity of anthropogenic activities have challenged the ability to secure water resource sustainability (Yang *et al.*, 2019). Climate change induces higher temperatures resulting in a direct effect on vectors and the spread of disease. These increased temperatures will change the survival conditions of several human pathogens, thereby allowing the infiltration of new vectors and diseases (Schernewski *et al.*, 2014).

Wastewaters are known to contain large quantities of enteric viruses (Adriaenssens *et al.*, 2021). Wastewater treatment plants (WWTPs) are not constructed such that they specifically eliminate enteric viruses from effluents. These effluents flow into rivers which may be used for varying purposes, be it domestic or recreational. To identify viral sources of contamination and to understand virus epidemiology, it is necessary to monitor viruses that are circulating in the environment (Prevost *et al.*, 2016).

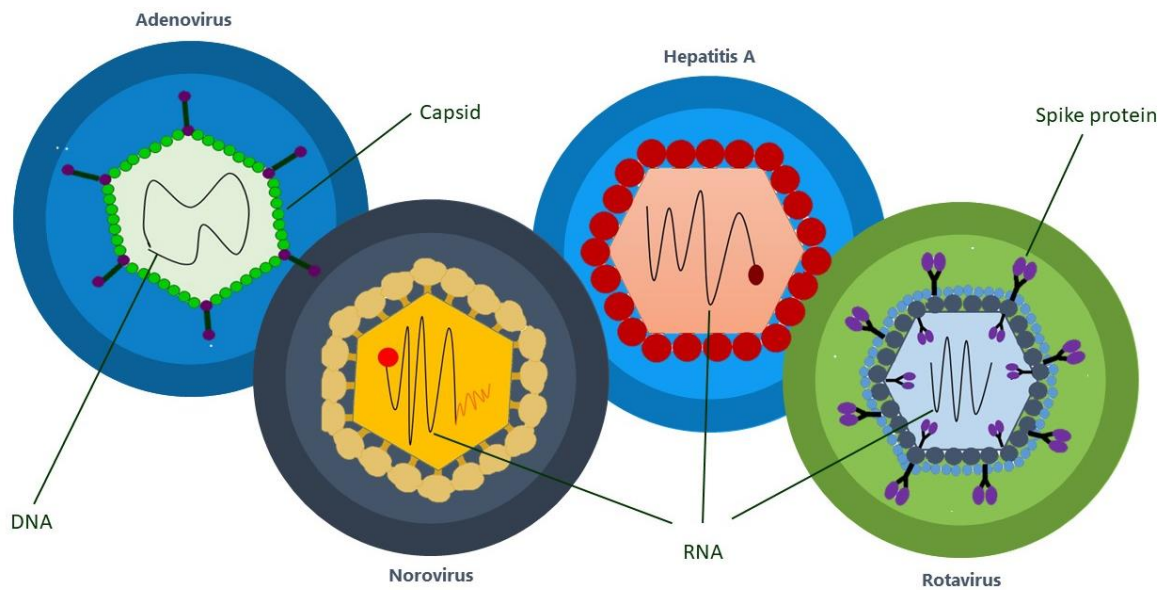


Figure 1. Depiction of two-dimensional structures of waterborne virus of the most common enteric viruses (Adapted from Altintas *et al.*, 2015)

2.3.1 Distribution and survival of waterborne viruses in a riverine environment

Human enteric viruses are extremely stable in the environment and can persist for long periods in the water. This is because they lack a lipid envelope (Prez *et al.*, 2015) as seen in Figure 1 above. Enteric viruses are considered emerging waterborne pathogens as they can cause disease after ingestion even with a low infectious dose (Prevost *et al.*, 2015). According to research, the intestine serves as a complicated interface between enteric viruses and the host, affecting both susceptibility and immunity to infection. When enteric viruses travel through the gastrointestinal tract to infect their hosts, they are exposed to a variety of environments. Numerous elements of virus biology, including how effectively they may infect their hosts, can be significantly impacted by this relationship. The fact that enteric viruses are typically non-enveloped is probably due to the harsh environment of the digestive tract, which disrupts a lipid membrane (Lockhart *et al.*, 2022). Therefore, a lipid membrane would not last long in a waterborne environment hence only non-enveloped viruses will survive. Their cellular and molecular structures allow them to be more resistant to conventional water treatment techniques. When viruses were exposed to UV light, it was believed that this would cause

damage to the viral genome, whereas chemical oxidants such as free chlorine would cause damage to the viral capsid proteins. Unlike amino acids, nucleotides are known to be more reactive to UV light. UV radiation can, however, also cause damage to viral proteins, indicating that it employs a variety of mechanisms for virus disinfection. The reaction rates of chemical oxidants such as free chlorine with amino acids are normally higher, yet it has been demonstrated that they can damage viral proteins as well as genomes (Gall *et al.*, 2015). In rotaviruses, a three-layered capsid protein and double-stranded RNA are thought to contribute to their resistance. The viral structure affects how resistant viruses are to inactivation processes. It has been demonstrated that single-stranded viruses are typically more vulnerable to UV radiation than double-stranded viruses, such as human adenovirus and rotavirus. The ability of double-stranded viruses to repair their genomes during host cell replication is thought to be the cause of this behaviour (Corpuz *et al.*, 2020). Most viruses persist at lower temperatures as compared to higher temperatures. The persistence of enteric viruses in water can increase with virus adsorption to particulate matter present in the water (Bosch *et al.*, 2007).

It is important to understand the sources of pollution to prevent contamination in surface water. Various molecular methods can be used to differentiate between the sources of indicator bacteria in environmental waters using source tracking markers (Mohapatra *et al.*, 2007). The survival of viruses in the environment depends on the virus type as well as the type of environmental conditions that the virus is exposed to (Labadie *et al.*, 2020).

Due to enteric viruses' low infectious doses and longevity in the environment, outbreaks of these diseases could happen far from the source of contamination (Farkas *et al.*, 2018). Lower river flow indicates a lower rate of viral input dilution (Robins *et al.*, 2019). In consequence, when the river is used, virus concentration rises, posing a risk to the public's health.

Factors that influence virus survival in water include temperature, exposure to UV light, flow conditions, inactivation by surrounding micro-organisms and adsorption to and settling out or resuspension in sediments (Rusiñol, 2023; Opere *et al.*, 2020; Pinon and Vialette, 2019).

Faecal pollution affects the quality of sediments and soil which are known to contain more harmful organisms than water, such as enteric viruses. (Staggemeier *et al.*, 2015). Viruses found in suspension with particulate matter or in solid matrices remain viable for longer periods than in the water column (Schernewski and Jülich, 2001). Viral particle retention is related to pH, temperature, moisture content, hydrophobicity and isoelectric point (Staggemeier *et al.*, 2015). Absorbed viruses have been suggested to offer less surface for interaction with substances that may inactivate substances, thus protecting the viral structure (Rao *et al.*, 1986). Viruses also accumulate in sediments and can be ingested and concentrated by shellfish resulting in a possible foodborne outbreak due to consumption of the shellfish (Farkas *et al.*, 2018).

Viruses can survive for long periods in environmental waters due to their small size. However, their survival in surface waters is much shorter than in groundwater systems based on smaller temperature fluctuations and exposure to sunlight that acts as a disinfectant (Meixell *et al.*, 2013). Surface waters are exposed to seasonal flow fluctuations where there is decreased water flow during winter in KZN. This can contribute to viral prevalence due to the low removal rate of viral particles (Fernández *et al.*, 2012; Nilsson *et al.*, 2008). Waterborne disease outbreaks due to the presence of enteric viruses urge the need for a better understanding of the fate as well as transport mechanisms of these viruses from source to point of contamination and exposure in the environment (Farkas *et al.*, 2018).

2.3.2 Viral indicators of water quality

Faecal indicator bacteria are excreted at high concentrations as compared to waterborne pathogens. However, faecal indicator bacteria are unreliable as indicators of pathogens due to complex climatic and hydrological parameters which affect the survival of these pathogens within the estuaries. There is limited information available on how environmental variables affect the link between FIB and enteric pathogens (Henry *et al.*, 2016).

Ideal characteristics of viral indicators include high abundance, low removal and little to no seasonal variation. These viruses should also be able to be used as model viruses in tertiary treatment processes. Potential viral indicators should not reproduce within a given environment. They should display enhanced resistance against environmental stressors such as ultra-violet irradiation from sunlight and treatment processes as compared to FIB (Bofill-Mas *et al.*, 2013). Viral indicators should have an existing relationship that is established with a microbial pathogen where they have similar growth and survival patterns within the natural environment (Henry *et al.*, 2016). It must however be noted that several different viral pathogen indicators may be needed to better characterise an overall water resource health risk. A single water quality indicator may not give effective coverage, especially in different places and over time, as they may not always be ubiquitous, unlike *E. coli*. An index may be needed, which includes several viral and bacterial indicators, to give a better water risk rating.

Adenoviruses (AdVs) and enteroviruses (EVs) are two out of several viruses that have been identified as possible indicators of the presence of enteric viruses in water (Wong *et al.*, 2012). Adenovirus has shown high prevalence in different water matrices, seasons and river locations worldwide. This corroborates their use as a viral indicator with the potential to indicate the fate of viral pathogens and faecal contamination in water (Rusiñol *et al.*, 2015). Being a DNA virus, it has a lower mutation rate as compared to RNA viruses, allowing it to be detected using molecular techniques. Another advantage it has over RNA viruses is that it is easier to manipulate which therefore lowers the cost associated with it (Poma *et al.*, 2012).

Aichi virus (AiV), polyomavirus (PyV) and pepper mild mottle virus (PMMoV) have been suggested as useful viral indicators in water as they are consistently detected in influent and effluent wastewaters in high concentrations showing no seasonal variability and persistent throughout the wastewater treatment process (Kitajima *et al.*, 2014; Farkas *et al.*, 2020). It has also been previously suggested that PMMoV be used as a novel viral indicator in marine and river water for faecal pollution (Rosario *et al.*, 2009; Hamza *et al.*, 2011a).

Despite the fact that rotavirus has been proposed as a faecal indicator, there has been little to no link shown between rotavirus concentrations and FIB. It is therefore impractical to utilise it as an indicator organism (He *et al.*, 2008).

2.4 Sources of enteric viruses in freshwater environments

2.4.1 Invasive species and industrialisation

Invasive species and infectious diseases are becoming more widespread with the increased connectivity of the global population. Pathogens and disease vectors are spreading across continents due to human transport, climate change and changes in land usage. Novel zoonosis may also happen (e.g. bush meat or other exposure). Infections tend to occur in those areas where there is poor sanitation and crowded living conditions. Where there is improved hygiene and sanitation, infections are delayed or prevented. This allows more people to become susceptible to diseases as immunity may be limited or absent. Under these circumstances, epidemics can occur from the contamination of one source such as a river (Gullón *et al.*, 2017).

To fully understand the effect and impact of disease and invasive species, observational and experimental approaches are necessary. These approaches will also help us to understand the biotic and abiotic stressors which will enhance or reduce their effects (Schade *et al.*, 2019; Crowl *et al.*, 2008). Invasive alien species are said to be the symptoms of constantly changing natural environments due to drivers such as climate change, increased urbanisation and various other land-usage changes (Bonanno, 2016). Invasion begins when a pathogen moves from its native range into a novel habitat as seen in Figure 4 below. After arriving, they might not survive, endure but be unable to spread or start reproducing and growing their local distribution. The pathogen starts to broaden its distribution as the population grows, leading

to several distinct, increasing populations on a continental and regional scale.

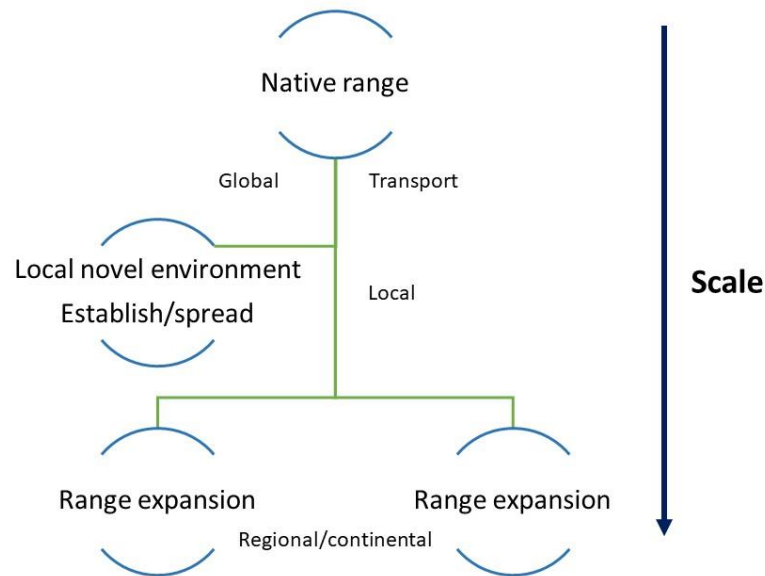


Figure 2. Invasion sequence of pathogens via a transport mechanism from their native range to a novel habitat on a global scale. (Adapted from (Crowl *et al.*, 2008).

2.4.2 Soil erosion

Viruses found in suspension with particulate matter or solid matrices remain viable for longer periods than if they were in the water (Schernewski & Jülich, 2001). Adsorption to or resuspension in sediments influences virus survival. The quality of sediments and soil is affected by faecal contamination. These matrices are known to harbour more pathogenic organisms than water, including enteric viruses (Staggemeier *et al.*, 2015). Adsorbed viruses have been suggested to offer less surface for interaction with substances that may cause inactivation, thus protecting the viral structure (Rao *et al.*, 1986).

Viruses found in the soil can also enter the groundwater system. They can remain suspended in surface waters and be transported via the current or deposited into the particulate matter in sediments, after which, with high rainfall or extreme weather conditions, they can be re-suspended (Madoux-Humery *et al.*, 2016). However, adsorption to particulates and sedimentation without resuspension (e.g. in some impoundment sediments, or if there is a

further capping sediment layer) does of course provide one removal mechanism. Viruses also accumulate in sediments and can be ingested by shellfish resulting in possible foodborne outbreaks (Farkas *et al.*, 2018). Virus particle shedding depends on the type of virus and the surface charge of the virion. Virus particle retention in the soil is related to the type of soil, pH, temperature, moisture content, hydrophobicity and isoelectric point (Staggemeier *et al.*, 2015). Viruses from pasturelands can be introduced into rivers through high catchment rainfall runoff/erosion or landslides where landslides that contain contaminated material can transport pollutants directly from the land into the river. A method for risk estimation associated with contaminated soil entering the river network was suggested and it can also be used in river basin management to estimate contaminant load from sediments (Göransson *et al.*, 2012; Göransson *et al.*, 2014).

2.4.3 Inadequate sanitation and broken/blocked/surcharging sewers

Any form of sanitation may become compromised if the sewage network is affected in any way i.e. pit latrines may become overfull or washed out by high-intensity rain events, and waterborne sanitation in particular may deliver faecal contamination directly to watercourses. Undersized or old/poorly designed pipework, blockage problems, lack of maintenance and stormwater ingress can all lead to surcharges or direct discharges, and as most sewers use gravity, they are mostly placed alongside watercourses. Therefore, faecal contamination can rapidly enter natural water resources. As there is no form of treatment or removal other than from natural processes, these types of issues may result in extremely high viral concentrations in the receiving waters. In the case of Pietermaritzburg, estimated direct sewer spills exceed 20% of the volume of sewage that should routinely be reaching the sewage treatment plant. In other nearby towns, that estimated sewer system loss is much higher, and at times (sometimes for extended periods) is 100%.

2.4.4 Inadequate wastewater treatment

Efficient wastewater treatment processes are vital for the protection of public health against outbreaks of enteric microbial infections (Poma *et al.*, 2012). Treated wastewater can become a source of reusable water, fertiliser, soil conditioner and energy supply. However, if this water is inadequately treated, it could limit its agricultural use, such as irrigation water for crops for instance (Peña *et al.*, 2019).

A functional wastewater treatment process will remove large numbers of virus particles, even though it may not inactivate them much during the treatment process, but the treated effluent at the end of the main process will still contain significant numbers of virus particles. Wastewater plants experiencing treatment problems are far more likely to discharge very high loads of viruses in their effluent stream.

Ultraviolet light (UV) disinfection is currently accepted as one of the disinfection processes available for potable water and can be used in wastewater disinfection if the effluent is of good quality to start with (and if the power supply is consistent). This involves irradiating the water using low-pressure monochromatic UV radiation (UVC). The portion of the energy spectrum from 200 to 310 nm is where UV light is most effective at destroying microorganisms. UV radiation is typically used at water treatment facilities in the 250 to 270 nm wavelength range (Askenaizer, 2003). This process causes damage to microbial pathogen genomes by producing thymine photodimers amongst adjacent pyrimidine nucleotides where nucleic acids (DNA and RNA) are damaged as well as proteins. This action inhibits genome replication or transcription and alters the surface protein structure. Therefore, microbes are prevented from replicating and causing infection (Wigginton *et al.*, 2012). This type of water treatment process does not produce any harmful by-products as is seen when using chlorine and ozone as disinfectants. Also, there are no residual protective effects left in the water once treated (Guerrero-Latorre *et al.*, 2016).

Ozone treatment is used as an alternative for the removal of microbial contaminants in sewage. This treatment has been effective in the removal of enteric viruses as well as bacteriophages (Wang *et al.*, 2018). However, the inactivation of viruses in wastewater through ozonation may be inhibited due to the high amounts of organic material present in the water. A mode of action utilised through ozone treatment is a conformation change of the viral capsid proteins by oxidation. Alteration of these proteins results in the capsid being destroyed or the host/virus-cell receptor binding process being suppressed (Shannon *et al.*, 2008).

Adenoviruses have shown resistance to ozone treatment. However, other viruses have shown susceptibility to ozonation due to a decrease in the overall number of viral genomes suggesting that the treatment may not only change the viral capsid conformation but could potentially open or destroy some virus capsids thus exposing the viral genome to nucleases present in the surrounding environment (Wang *et al.*, 2018).

Most enteric viruses are resistant to UV light and chemical disinfection. Viruses can be challenging to remove from water due to their small size and resistance to conventional disinfection processes. Numerous factors might affect disinfection, including water, pH, temperature, the type of micro-organism present, the type of disinfection used, the amount of disinfectant used, contact time, and the inorganic and organic content of the water (Lanrewaju *et al.*, 2022). Traditional disinfection using chlorine is substantially effective when using large doses to inactivate viruses. However, dangerous disinfection by-products (DBPs) are generated simultaneously (Zheng *et al.*, 2015). Chlorine is the most common disinfectant used due to its low cost, high efficiency and residual biocidal impact (Zuo *et al.*, 2015).

The advantages of UV disinfection as compared to chemical disinfection, such as chlorination and ozonation, are there are no additions of chemicals, no residual, nominal DBPs, UV is non-corrosive in nature and there is ease of operation (Qiu *et al.*, 2018). Photoreactivation could pose a potential challenge when using UV disinfection techniques. Therefore, photocatalysis could be as effective as chlorine for the inactivation of viruses in water, simultaneously limiting DBP formation and utilising less energy than UV radiation (Zheng *et al.*, 2015).

2.4.5 Influence of recreational activity on viral loading

Generally, rivers dilute and remove effluents, which are a result of human activity. Understanding of how viruses behave in aquatic systems is however unclear due to a lack of specific laws in place for viral monitoring of water resources. Contamination of recreational waters by human viral pathogens is a concern for athletes participating in water sports, as they may fall ill due to the presence of waterborne pathogens (Staggemeier *et al.*, 2017). Recreational exposure to pathogen-polluted waters can occur in swimmers and their subsequent diseases are commonly termed as recreational waterborne illness (RWI) where gastroenteritis is known as the most common illness. Viruses, bacteria or parasites can cause gastroenteritis. Person-to-person contact, such as touching contaminated hands, vomit, faeces, or drinking tainted water, can spread viral gastroenteritis. Ingestion of contaminated water is the main mode of pathogen exposure, and it can result in swimming-related gastroenteritis. The USEPA has identified body-contact recreational activities and swimming as potential sources of microbial contamination in recreational waters. Recreational users may also directly contribute to the pathogen load of the water resource.

The population is continuously exposed to a range of different disease-causing micro-organisms in recreational waters. The sources of faecal contamination originate from both animals and humans. However, there is a higher risk involved with contamination of human origin due to the presence and larger load of human-specific enteric viruses. Therefore, it is of great importance to identify potential contamination sources of recreational water for suitable mitigation strategies to be put in place. By doing so, the risks posed to recreational water users can be accurately assessed (Ahmed *et al.*, 2018).

Much of the hazards that recreational water users are exposed to are instantaneous in nature where accidents or exposure to infectious doses of micro-organisms occur within a short period. Ongoing resource contamination problems such as long-term unresolved sewer problems, may however mean that the resource is always highly contaminated, hence any exposure may result in infection.

2.5 Factors contributing to the survival of waterborne enteric viruses in a freshwater environment

Freshwater ecosystems are comprised of rivers, lakes, ponds, wetlands, groundwater and reservoirs/dams. The survival of viruses in the environment depends on the virus type as well as the environmental conditions that a particular type of virus is exposed to. Factors such as temperature, pH, rainfall and anthropogenic stresses are known to affect the survival of enteric viruses in freshwater habitats. The major factors are discussed below.

2.5.1 Rainfall patterns

Rainfall events can create combined sewer overflows (or excessive stormwater ingress to sewers in the case of RSA) thereby introducing various sewage-borne contaminants into aquatic environments, including waterborne pathogens, organic contaminants, suspended solids, heavy metals and chemical compounds (Hata *et al.*, 2014). When there are periods of heavy rainfall, potable water quality can also be severely compromised through cross-contamination of water sources. This can be due to inflow and infiltration between water pipes and sewage (Gullón *et al.*, 2017). The highest natural water resource viral concentrations have been reported in dry periods where there is minimal rainfall and low river flow, which thereby reduces the dilution of treated effluents or sewer spills by riverine systems, resulting in higher pathogen concentrations per litre of water. Therefore drought may also represent a decrease in the quality of water where the die-off of microbial pollutants in the environment would be higher under low-flow circumstances (Rusiñol *et al.*, 2014; Rinaldo *et al.*, 2018). This also leads to a potential increase in these concentrations in wet seasons due to sporadic sewage overflows during recurrent storm events (Rusiñol *et al.*, 2015).

2.5.2 Temperature

Environmental conditions of a river, such as exposure times to UV light and temperature, are subject to change daily, thus resulting in changes in the variability of virus survival (Corsi *et al.*, 2014). Climate change induces higher temperatures, as well as potentially increasing the

likelihood of floods and droughts, resulting in a direct effect on vectors and the spread of disease. These increased temperatures will change the survival conditions of several human pathogens, thereby allowing the infiltration of new vectors and diseases. Even slight climate changes can intensify or contribute to the emergence of human health issues (Schernewski *et al.*, 2014; Lyth and Holbrook, 2015).

The effect of temperature on viral persistence can be linked to protein denaturation, microbial or enzymatic activity as well as RNA or DNA damage. Viruses are resistant to high temperatures, between 75 to 95°C (Sauerbrei and Wutzler, 2009; Belon and Frick, 2011). However, there is better survival of enteric viruses at low temperatures, between 20 to 30°C. This is because high temperatures can damage the viral capsid and genetic material (Dias *et al.*, 2018). However, some viruses such as hepatitis A and parvovirus demonstrate high thermal resistance (Bosch *et al.*, 2007).

Various researchers have studied the effect of temperature on the distribution of enteric viruses in different aquatic habitats. Hepatitis A virus is considered to be environmentally stable, seeing as while it has a 100-fold decline in infectivity over a period of 4 weeks at room temperature, this may take between 3 to 10 months in water which is a major public health risk (Cormier and Janes, 2016). Viruses can survive for long periods in environmental waters due to their small size. However, their survival in surface waters is much shorter than in groundwater systems based on temperature and exposure to sunlight (Meixell *et al.*, 2013).

2.5.3 Weather-related disasters

Global climate change is anticipated to affect the intensity, occurrence and extent of extreme weather events such as storm surges, floods, excessive precipitation and drought. However, these events can impact the quality of water through managerial and physical stresses (Gullón *et al.*, 2017). The risk of river flooding is said to increase soon, where a higher discharge results in increased transport velocity or river flow rates. The transport distance of human enteric pathogens depends on the flow velocity as well as the die-off or inactivation rates of

these micro-organisms. Microbiological contamination occurs during single events. They can rarely be predicted and require rapid action to be taken (Schernewski *et al.*, 2014; Jovanovic *et al.*, 2017). Seasonal flooding and rainy periods can dilute the amount of faecal contamination and viral markers present in environmental waters. This is due to the increased flow. It could still represent a microbiological risk to the surrounding community and overall population. A reduction in river flow means there is a lower dilution rate of viral input. In turn, there is an increase in viral contamination when the river discharge enters the sea resulting in a public health risk.

2.5.4 Anthropogenic stressors

Surface water is continuously contaminated with human enteric viruses via sewage and other faecal waste sources. There has been a significant anthropic impact on water resources as well as the environment. The concentration of pathogens in these waters is of significant spatial and temporal variability. Zones that have been associated with body-contact recreation have shown the highest concentration of pathogens (Hoyer *et al.*, 2015).

Human adenoviruses have been commonly associated with anthropogenic activities due to their high incidence rate in water samples. Cases and outbreaks are often underestimated due to a lack of reporting of infections. Enteric viruses are one of the main etiological agents responsible for approximately 90% of confirmed cases. Recreational waters are an indicator of tourism development where the most common cause of outbreaks is said to be faecal contamination by an infected person (Hoyer *et al.*, 2015; Federigi *et al.*, 2017).

2.5.5 Lack of source water protection

Recreational waters do not undergo any treatment and are considered to be suitable for swimming under specific bacterial levels. The presence of viruses in these waters is a potential risk to the public and cannot be ignored (Prez *et al.*, 2015). Viruses can enter surface waters via septic systems, treated and partially treated wastewater effluents, sewer line spills and combined sewer overflows. Viruses that are present in land-applied manure or sewage sludge

disposal can also enter surface waters (Fong and Lipp, 2005). They can remain suspended in surface waters and transported via the current or be deposited into particulate matter in sediments and thereafter be resuspended with high rainfall or extreme weather conditions (Madoux-Humery *et al.*, 2016). Rapid exposure to sunlight and elevated oxygenation during turbulent discharge can cause a reduced prevalence of pathogens in surface waters (Partyka *et al.*, 2018).

Microbial source tracking (MST) utilises a combination of methods that identify the principal sources of contamination (Fong and Lipp, 2005). It plays a vital role in remediation against particular sources of pollution. Molecular markers derived from prokaryotes, eukaryotes and viruses are target sequences obtained directly from the host or micro-organisms associated with the host and are used in MST. This however comes with limitations such as the lack of total host specificity in animal and human microbial markers, lack of temporal stability in various host groups and the fact that markers not originating from faecal sources do exist (Roslev and Bukh, 2011).

Viral contamination of receiving/river water is considered an etiological issue due to wastewater treatment plant effluents entering the water upstream and the same water supplying potable water treatment plants downstream (Kishida *et al.*, 2012).

2.5.6 Water distribution system malfunction

Understanding the relationship that exists between FIB, pathogens and environmental factors is essential for assessing the dynamics and risks associated with faecal pollution in environmental waters (Henry *et al.*, 2016). Public water supplies are expected to be managed with extreme weather events. However, these events can impact the quality of water through managerial and physical stresses. In addition, older distribution and water treatment systems are more likely subject to problems arising from heavy rainfall events. This increases the susceptibility of potable water supplies (Gullón *et al.*, 2017).

Currently, no standards or regulations are enforced to monitor viral concentrations in potable water in RSA except the general log reduction requirement enforced during the treatment of surface and groundwater used as potable water sources. The USEPA Surface Water Treatment Rule (SWTR) includes a virus treatment requirement that states that the treatment used should be sufficient to inactivate or remove 99.99% (4 log) of viruses. This requirement is based on the acceptable level of illness in a community, which is said to be one case per 10 000 consumers and the most probable level of viruses present in surface water. This rule highlights the importance of monitoring potable and surface water for viruses and the underlying risk of not monitoring surface waters.

2.5.7 Enteric virus inactivation

The viral genome and the capsid, which contains the virus-coded protein, are the two fundamental parts of a virus. Viruses can be classified into two groups, namely enveloped and non-enveloped viruses.

As they do not always cause cell lysis upon cell exit, enveloped viruses are regarded as being less virulent, even if cell death frequently occurs as a result of viral replication. The capsid is encased by an outer membrane in viruses that are enveloped. The envelope is made up of a lipid bilayer which contains viral proteins. These proteins are vital for binding with host cells. These viruses can exploit the membrane of the host cell to assemble their envelope, or outer membrane, during virus assembly and escape from the host cells. By doing so, the viruses can evade the immune systems of their hosts and avoid cell lysis. It is known that enveloped viruses are more sensitive to pH, heat, dryness, and basic disinfectants. Some viral envelopes assist in determining viral stability characteristics such as being less susceptible to chemical or physical inactivation.

Viruses that lack an envelope are more virulent because they lyse host cells. Since they lack the additional lipid membrane, cell lysis is the most frequent exit from the host cell. During this process, the viruses compromise the cell membrane's integrity, leading to cell death and

severe tissue damage in the host organism. Non-enveloped viruses are more resistant to pH, heat, dryness and disinfectants and can survive in the gastrointestinal tract of mammals as well as tolerate harsh environmental conditions.

Various factors influence enteric virus survival in the environment namely chemical, biological and physical. To inactivate a virus, several methods of inactivation concentrate on one or more of its functionalities. The genome is typically rendered incapable of replication after exposure to UV, singlet oxygen, and hypochlorous acid, whereas chlorine dioxide and heat prevent host-cell recognition and binding (Wigginton *et al.*, 2012). Water matrix has an impact on virus inactivation rates as well (Ge *et al.*, 2021). Viral inactivation is caused by environmental stimuli acting directly or indirectly on the viral genome, capsid, or envelope, if present. Additionally, viral populations have defense mechanisms that usually entail physical protection from harmful consequences. These defensive behaviours include aggregation, adhesion, or internalisation within biological structures (Pinon and Vialette, 2019).

Viral survival in the environment is primarily impacted by increased temperature, which can cause capsid dissociation, protein denaturation and nucleic acid degradation. Aggregation, where viral particles form aggregates in liquid suspension, is a critical component in viral resistance to damaging environmental conditions.

2.6 Emerging technologies in enteric virus detection from freshwater environments

2.6.1 Conventional methods

Viral detection involves numerous steps, including concentration, extraction and detection or quantification. The most popular techniques for identifying virus particles in water include filtration, ultrafiltration, ultracentrifugation, and flocculation (Hrdy and Vasickova, 2022). Quantification of enteric viruses present in surface and environmental waters can be a challenging task due to the complex nature of the matrix in which the organisms are suspended (Schijven *et al.*, 2019). Human enteric viruses are present in low concentrations

in environmental waters, therefore large amounts of water need to be concentrated beforehand to obtain detectable levels of viruses that may be present in diluted form. In addition, virus concentration from environmental waters is affected by temperature, turbidity, organic matter and salinity (McMinn *et al.*, 2016).

The enrichment of viruses from environmental water has been achieved using a variety of techniques. These include adsorption/elution methods (electronegative membrane, electropositive membrane, electronegative cartridges, electropositive cartridges, glass wool and glass powder), entrapment ultrafiltration (alginate membranes, single membranes and hollow fibres), ultracentrifugation and hydroextraction.

Challenges associated with conventional virus detection techniques include filter clogging and the removal of infective viruses adsorbed to particulates thereof, insufficient reliable standard methods for complex matrices such as river water and wastewater, low recovery rates, sample pre-treatment and excessive sample volumes. Tissue culture has been considered the gold standard for the detection of infectious viruses. However, detection using cell culture assays can be time-consuming and resource and labour-intensive. Infectivity assays are sometimes not available for virus detection in environmental waters where *in vitro* cell culture systems have not been established for most enteric viruses (Ryu *et al.*, 2018; Lee *et al.*, 2013; Cormier and Janes, 2016; Farkas *et al.*, 2018; Pang *et al.*, 2012).

Currently, the legislation does not include virus monitoring for environmental waters. This can be attributed to the cost of performing analytical methods routinely, challenges in standardising methodology and the reliability of results where the viability of viruses detected cannot be established (Girardi *et al.*, 2019).

2.6.2 Molecular methods

2.6.2.1 Quantitative real-time PCR (qPCR) and reverse transcription PCR (RT-qPCR)

Virus identification in ambient water samples can be done using a wide variety of analytical techniques. To analyze viral contamination in water, molecular techniques like PCR and quantitative real-time PCR (qPCR) are most frequently utilised. These techniques have the highest sensitivity and specificity (Hamza *et al.*, 2011b).

Two regulatory standards have been proposed for the detection of viruses, namely ISO/TS 15216-1 and 2, 2013. These methods are based on real-time reverse transcription PCR (RT-qPCR) which has become the gold standard for virus quantification (Lowther *et al.*, 2019). These particular techniques are faster and more reliable with enhanced sensitivity as compared to conventional detection methods. This method relies on the generation of a standard curve, which necessitates sensitive calibration and consistent reference material. An entire population of targeted enteric viruses can be detected using RT-qPCR including non-infectious viruses using specific primers and probes (Ryu *et al.*, 2018).

Using PCR to detect viruses has its shortcomings since virus viability cannot be determined. Another factor to take into consideration is that there are low viral concentrations present in environmental waters and inhibitory substances in these environmental waters may also give false negative results when using PCR (Fout *et al.*, 2003). The virus may not be infectious because the genome segments that PCR is targeting may be relatively unaffected by environmental factors. Numerous studies have shown that qPCR methods routinely overestimate virus survival. While qPCR may be useful to determine virus presence in the environment, it may not be able to evaluate the effect of environmental conditions on the survival of viruses or the risk associated with water consumers (Pinon and Vialette, 2019).

2.6.2.2 Multiplex PCR (mPCR)

Cost and occasionally a lack of sufficient test volume samples limit the use of PCR in routine laboratories. The multiplex PCR assay was developed to address these limitations and enhance the detection of PCR. However, its application for the detection of enteric viruses in environmental waters is limited (Formiga-Cruz *et al.*, 2005).

Multiplex PCR can be used to simultaneously detect more than one target gene in a single reaction using different primer sets. Despite its application in many areas, the sensitivity of mPCR is reduced due to the interference of primer sets. This can however be avoided by optimising the primer design. Using multiplex qPCR, which utilises different reporter dyes, can support specific detection of simultaneous targets. However, there are a limited number of fluorescent reporter dyes and channels in PCR instruments (Huang *et al.*, 2011). Two multiplex RT-qPCR assays were validated for the detection and quantification of viruses from sediment samples (Farkas *et al.*, 2017). Another study successfully detected adenovirus, norovirus and rotavirus in river water, groundwater and wastewater using a multiplex RT-qPCR assay (Lee *et al.*, 2016).

2.6.2.3 Digital PCR (dPCR)

Digital PCR (dPCR) is known for its sensitivity and accurate absolute quantification (Mao *et al.*, 2019). This allows one to determine the number of target copies without generating a standard curve. This method partitions a sample into individual reactions either on microfluidic chips or in micro-droplets. Each reaction will then contain either a single copy or no copies of the target DNA or RNA sequence. The signal generated in dPCR is then measured after amplification and is also known as an endpoint approach. The absolute amount of nucleic acid target is calculated using a Poisson distribution model. This novel technology has been used to detect rotavirus in surface waters (Rački *et al.*, 2014; Coudray-Meunier *et al.*, 2015), coronavirus (Vasudevan *et al.*, 2021), hepatitis A and norovirus GI and GII from water samples (Han *et al.*, 2022).

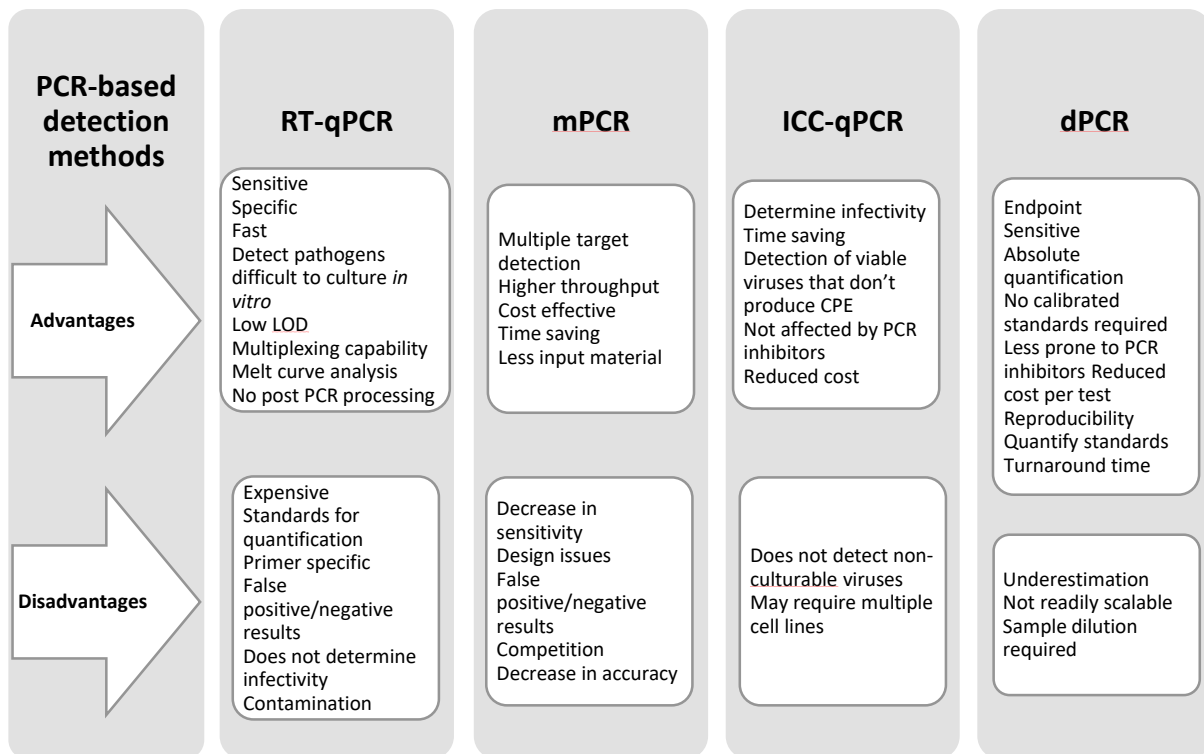


Figure 3. Advantages and disadvantages of PCR-based methods for enteric virus detection

2.6.2.4 Flow virometry

Our knowledge of cells, immunology, and microbiology has been greatly improved over the past few decades thanks to flow cytometry, which has revolutionized the area of biology. Recently, flow cytometry gave rise to flow virometry, a new technique for identifying, analyzing, and characterizing individual viral particles. The development of modern flow cytometers, as well as calibration and tuning techniques, have made it possible to detect viruses using this technique (Zamora and Aguilar, 2018).

To detect nucleic acids, DNA is amplified using a biotin-labeled primer. The amplicons are hybridised to capture probes that are bound to microspheres known as beads. The microspheres are filled with various concentrations of an infrared dye and a red dye. This creates 100 beads and each bead has a unique spectral identity. This method can therefore detect up to 100 analytes in a single microplate well. Microfluidics is used to control the flow of microspheres between two lasers with differing wavelengths. This technology can be used in multiplex for the detection of viral pathogens in wastewater (Hamza *et al.*, 2014).

Flow virometry could be utilised as a tool for numerous applications. Applications include virus enumeration, environmental viral population discrimination or the determination of virus glycoprotein and lipid topography of viral particles. It has been used to quantify viruses from lake water and activated sludge samples and thereafter different viral populations within those samples were determined. Therefore, flow virometry can be a helpful method for researching and finding new viruses in natural bodies of water or for ensuring water quality.

2.6.2.5 Loop-mediated isothermal amplification (LAMP)

An isothermal nucleic acid amplification technique called the LAMP assay typically uses a series of four or six distinct primers that bind to complementary sequences on the molecular target. The amplification process just needs a steady temperature. Rapidity, affordability, high sensitivity, and high specificity are only a few of its many advantages over RT-qPCR. Colour-based reporters that can be added to the reaction mixture make the obtained results easily readable with the unaided eye (Je *et al.*, 2020; Hang *et al.*, 2005).

2.6.3 Combined approaches

Chlorine and heat treatment are commonly used to disinfect water. Survival of viruses against disinfection is due to numerous factors such as the mechanical stability or plasticity of the viral capsid, the level of secondary structure present and the level of interaction and protection by viral capsids. Heat exposure can destroy the viral capsid whereas chlorine and ozone treatment can damage the viral capsid and nucleic acid depending on the dose (Prevost *et al.*, 2016). However, the efficiency of these disinfection techniques cannot be accurately assessed due to molecular tests not having the capability to distinguish between inactivated and infectious virus particles. To overcome these limitations many studies use fluorescent intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA). These dyes assist with the differentiation of potentially infectious and dead virus particles which are detected in samples (Elizaquível *et al.*, 2014).

There are two theories associated with the use of these intercalating dyes, one being that a virus containing a damaged capsid is not infectious and two, that intercalating dyes can gain access and bind to genomes to specifically block the amplification of damaged particles (Prevost *et al.*, 2016). These particular dyes enter the viral capsid of dead virion particles and bind to the double-stranded nucleic acid present. This occurs after a photo-activation step, which prevents RNA retrotranscription and additional amplification by RT-qPCR as shown in Figure 6 below. The effectiveness of using EMA or PMA is dependent on the target virus and the disinfection treatment used (Parshionikar *et al.*, 2010; Kim *et al.*, 2011; Karim *et al.*, 2015; Fuster *et al.*, 2016; Leifels *et al.*, 2015). However, PMA requires structures or double-stranded nucleic acids for binding. Binding may be less efficient when heat is used as the method of inactivation due to the denaturation of stable secondary structures that are present within the genome. It has been demonstrated that PMA-RT-qPCR assays cannot entirely inhibit the amplification and detection of inactivated viruses. There is also the likelihood that viral capsids could undergo structural modifications which can cause them to be non-infectious. This can occur without causing cavities which intercalator molecules can use to gain access to the nucleic acid (Fuster *et al.*, 2016).

The detection of viral genomes does not imply the infectivity of that particular virus. Therefore, molecular approaches need to be further verified to predict the infectivity of a virus in environmental waters by combining with other methods (Fuster *et al.*, 2016). For example, cell culture methods can be incorporated with RT-qPCR to develop a method that is sensitive, quantitative and able to determine the infectivity of a virus. These are known as integrated cell culture PCR (ICC-PCR) (Pang *et al.*, 2012; Lee *et al.*, 2013). It can be used in virus disinfection studies to determine the inactivation rate of viruses (Ryu *et al.*, 2018). This technique has certain disadvantages, such as the sample volume size needing to remain relatively small. Also, it requires specific and optimal primer sets for different enteric viral groups.

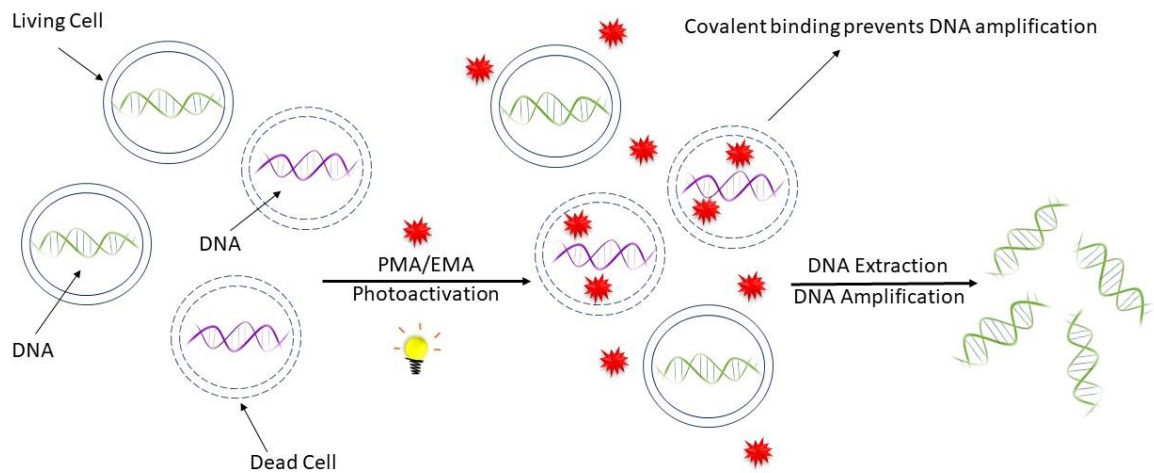


Figure 4. Mode of action used by EMA and PMA intercalating dyes

2.6.4 Other methods

2.6.4.1 Biosensors for the detection of enteric viruses

Biosensors can be used as an alternative to PCR-based detection methods. Utilising biosensors for virus detection offers many advantages such as rapid readings, turnaround time, specificity and inexpensive development (Altintas *et al.*, 2015). Biosensors are the fastest-developing technology for virus detection (Kleo *et al.*, 2011). It is becoming more popular due to its high sensitivity, rapid results and high portability. Pathogenic biosensors could be installed in wastewater pipelines to offer real-time data and online pathogen detection. Enteric viruses have been detected using electrochemical biosensors (Kadadou *et al.*, 2020). This is based on the detection of specific DNA sequences. Hybridisation occurs at the sensor surface which results in a quantitative signal being generated. Biosensors have an acceptable tolerance to inhibitory substances present in environmental samples. This technique has the potential for a reduction in cost and time as compared to other methods (Manzano *et al.*, 2018).

2.6.4.2 Metagenomics approach for differentiation of viruses

Metagenomics is becoming an increasingly popular technique for differentiating viruses such as norovirus, enterovirus, rotavirus and astrovirus (Schaeffer *et al.*, 2023; Strubbia *et al.*, 2019). It is a sequence-based analysis of a mixture of genetic material that relates to fungi, bacteria, viruses as well as humans. This material can be directly recovered from environmental waters. Metagenomics has the potential to be utilised in viral surveillance in environmental waters because of its sensitivity and broad scope of detection (Aarestrup and Koopmans, 2016). This influences high throughput next-generation sequencing (NGS) technologies.

Metagenomics is a powerful tool for characterising enteric viruses because viral particles can be isolated and sequenced directly from a sample without prior knowledge about the microbial communities present in that environment. Three main steps are making up the metagenomics approach, namely sample preparation, high throughput sequencing and bioinformatics analysis (Hamza and Bibby, 2019). The microbiological quality of potable water and wastewater can be assessed using genetic data from the virus as a whole rather than only viruses that are cultivable. Sample preparation for metagenomics remains the same as for PCR techniques (Aw *et al.*, 2014). Metagenomics of viruses does not focus on a particular virus. Consequently, there is a chance that the native virome of a sample will be detected. This could reduce the ability to identify some enteric pathogenic viruses, especially those that are present in low concentrations (Santiago-Rodriguez and Hollister, 2023).

2.6.5 Quantitative Microbial Risk Assessment (QMRA)

Environmental emissions such as effluents from sewage treatment plants can affect human health. For example, between 20% to 50% of community diarrheal disease has been due to unsafe drinking water where higher percentages are associated with waterborne diarrheal disease (Batabyal *et al.*, 2016). The impact of the environment on human health can be assessed through different environmental assessment methods. Another common approach

is quantitative risk assessment which can be utilised to determine the health risk to the surrounding community after exposure to pathogens and harmful chemicals (Kobayashi *et al.*, 2015; Li *et al.*, 2023; Liu *et al.*, 2021).

It is essential to monitor viruses circulating in the environment to aid in identifying sources of viral contamination and to create a better understanding of virus epidemiology (Prevost *et al.*, 2015). Risk assessments are used to predict the disease burden in the community, set microbiological standards for potable water supplies, identify cost-saving methods, determine the best treatment method with minimal DBPs and provide a framework that minimises the risk from water consumption.

To develop a risk reduction strategy, recreational activities need to be classified according to the degree of water contact one would experience during that activity. The degree of water contact directly influences the degree of contact with pathogenic micro-organisms. Therefore, the degree of contact can be classified into three categories as seen in Table 3 below.

Table 2. Degree of water contact during recreational activities (WHO, 2021)

| Degree of water contact | Description | Recreational activity |
|---------------------------|--|---|
| <i>No contact</i> | Recreational activity where there is normally no contact with water, or where water is incidental to the enjoyment of the activity | Angling from shore Sunbathing |
| <i>Incidental contact</i> | Recreational activity in which only the limbs are regularly wetted and in which greater contact (including swallowing of water) is unusual | Boating Fishing Wading |
| <i>Whole-body contact</i> | Recreational activity where the whole body or face and torso are frequently immersed, or the face is frequently wetted by spray, or where it is likely that some water will be swallowed | Swimming Diving Whitewater canoeing |

There have been no maximum acceptable values (MAVs) set for human viruses detected in potable water in many countries worldwide. The WHO has not made any recommendations in this regard. However, Canadian Health guidelines have implemented a maximum contaminant level goal (MCLG) of zero viruses in potable water. Much emphasis has been placed on viruses present in potable water however, there has not been much research done in

monitoring environmental waters for viruses. Many people use these waters for domestic and drinking purposes in low and middle income countries. Therefore, public health should be the primary focus when implementing water monitoring strategies. This was found to be evident during the coronavirus pandemic where SARS CoV2 was detected in surface water and wastewater (Maryam *et al.*, 2023). The monitoring of viral pathogens should ideally form part of the future classification of microbiological hazards in water using a combination of sanitary inspection and microbial risk assessment as seen in Figure 7. In doing so, it will be possible to address the risks and activate prevention schemes (Taylor *et al.*, 2001).

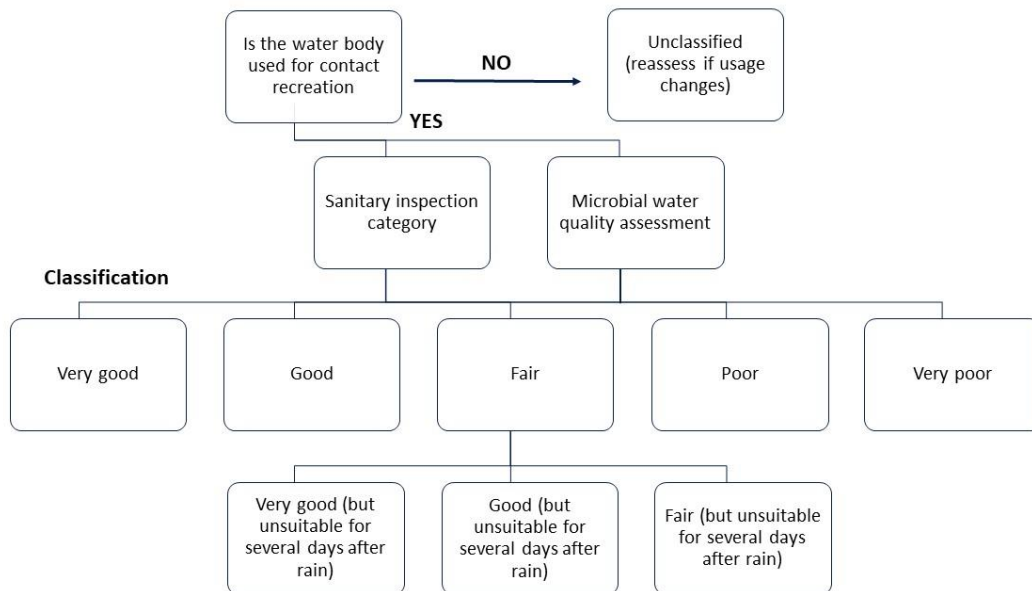


Figure 5. Framework used to assess recreational water environments (Adapted from WHO, 2013)

The WHO Guidelines for Drinking Water Quality (RS2, 2012) encourage the delivery of drinking water by implementing risk management strategies from catchment to consumer through the development of a Water Safety Plan (WSP). The WSP contains QMRA which is effective when applied correctly to assess a system’s ability to provide safe drinking water and to help identify key hazards, their sources and the treatment level required to achieve safe potable water (Petterson *et al.*, 2015; WHO, 2016). It is a probabilistic modeling technique

which is currently used to assess the public health risk associated with exposure to waterborne pathogens by direct or indirect ingestion of contaminated water from a specific location (Prez *et al.*, 2015; Burch *et al.*, 2021; Ahmed *et al.*, 2020). Specifically, risks associated with faecal pathogens in potable water and wastewater can be evaluated using QMRA. Further to this, one of the main advantages of using QMRA is that the probability of infection, as well as morbidity, can be quantified (Gwenzi and Siyawamwaya, 2022). Therefore, the protection of public health for recreational activities can be better managed. This can be achieved by implementing dose-response models which can estimate the risk associated with ingesting contaminated water during these activities. When developing a risk assessment model it is important to take into account the probability of infection, the duration of exposure, ingestion rates and the concentration of viruses present in the water (Kundu *et al.*, 2013).

The dose or number of micro-organisms that may cause infection depends on varying factors such as the specific pathogen, the form in which it is encountered, the exposure conditions and the host's susceptibility and immune status. The body seldom experiences a single remote encounter with a pathogen. In freshwater studies focused on the impact of faecal contamination on recreational water users, index bacteria including faecal streptococci and intestinal enterococci have been utilised to define water quality due to their behaviour being similar to the actual faecally derived pathogens (WHO, 2013).

The application of QMRA in water utilities and guidelines is restricted to enteric viruses whose properties make them a good representation of all related pathogenic viruses. This implies that if a reference waterborne virus can be controlled, then all other similar viruses that it represents can be controlled in the same manner. Water treatment is said to be effective if the index pathogen is removed. In the same way, if the reference virus is removed, the water would be considered safe for consumption (Schijven *et al.*, 2019).

Understanding the environmental fate of human pathogens in water is beneficial to minimise the public health risk (Rusiñol *et al.*, 2015). Quantitative microbial risk assessment of the risk of infection from recreational activities in water has provided great insight into the protection

of public health from a risk-based approach. Evidence from QMRAs confirms the lack of reliable water quality associations where the probability of contracting gastroenteritis illness was 8% for swimming and 13% for rowing or canoeing in rivers (Kistemann *et al.*, 2016).

Models combining the epidemiology of viral acute gastroenteritis and virus shedding and fate in sewers, WWTPs and rivers can be used to evaluate the health risks of different wastewater management scenarios and modulate tertiary treatments. The model accurately reflected seasonal variations in viral shedding and river contamination (Tesson *et al.*, 2019).

2.6.6 Early warning system for potential outbreaks

For the prevention and control of viral outbreaks, the sensitivity of infectious disease surveillance in rural areas should be enhanced. Management of rural environmental sanitation should be strengthened. Safe food and water should be provided in conjunction with improving the awareness of infectious disease prevention by enhancing health education (Yu *et al.*, 2015). Models have been developed to assess the effect of pathogen pathways and install early warning systems (Mälzer *et al.*, 2016; Tondera *et al.*, 2016). New legislation and viral quality standards will be needed as there is now more information on the viability and infectivity of viruses, especially after disinfection processes (Rusiñol *et al.*, 2015).

By quantifying the probability of infection, the public can be warned when contamination is expected to be at elevated levels as compared to the norm and more caution can be ensured when using these bodies of water. An early warning system can be developed to alert the public as to when contamination levels are excessive using QMRA models. Site-specific QMRA provides a better understanding of the risks presented by different contaminant sources resulting in estimates of which pathogens cause illness to recreational users in a specific body of water. When utilised efficiently, site-specific QMRA can be used as a safety mechanism for public health in recreational waters (Kundu *et al.*, 2013).

To be a valuable community partner in response to an outbreak situation, a healthcare establishment should be able to act quickly, rally efforts from various departments as well as

influence relationships with other community organisations. A stable relationship with health officials can ensure that efforts are appropriately targeted and resources are distributed (Duncan, 2018).

CHAPTER THREE – DETECTION OF PATHOGENIC ENTERIC VIRUSES AND THE EFFECTS OF ABIOTIC AND PHYSIOCHEMICAL FACTORS ON THEIR SURVIVAL IN A RIVERINE ENVIRONMENT

3.1 Introduction

Environmental water quality parameters of importance include changes in physical, chemical and biological parameters. Physical parameters include pH, temperature (°C), conductivity, turbidity, total dissolved solids (TDS) and dissolved oxygen (DO). Chemical parameters of potential significant importance may include heavy metals, phosphorous, alkalinity, silicate and nitrite concentrations (Opere *et al.*, 2020; Mugidde *et al.*, 2005). Biological water quality parameters include nutrients, algae, bacteria and viruses such as enteric viruses. Various factors can impact the persistence of viable enteric viruses in surface waters (Fong and Lipp, 2005; Paul *et al.*, 2021; Pinon and Vialette, 2019). One of the dominant abiotic factors affecting virus persistence is pH (Auffret *et al.*, 2019). Enteric viruses can survive in a broad pH range from approximately 3 to 10 at low temperatures for an extended period (Fong and Lipp, 2005).

The routine monitoring of waterborne enteric viruses is necessary for the protection of public health and water quality monitoring. When waterborne virus outbreaks occur, virus contamination levels are considered high at the source of the outbreak. The majority of enteric viruses cannot be easily cultured on cell lines, therefore molecular techniques such as PCR are used for their detection (Hamza *et al.*, 2011b; Bridle *et al.*, 2014; Gerba and Betancourt, 2019; Croci *et al.*, 2008). However, in large volumes of water, these viruses are diluted to as low as 1 to 10 infectious particles per litre of water (Gensberger and Kostić, 2013). Therefore, when analysing environmental samples, it is necessary to concentrate a large volume of sample before commencing with virus detection and enumeration. The most common concentration methods are carried out through filtration, ultracentrifugation and/or precipitation (Petrova *et al.*, 2020; Cashdollar and Wymer, 2013).

Researchers have employed a variety of PCR detection methods such as qPCR, RT-PCR and dPCR depending on the purpose of the study (Kadri, 2020). Quantitative PCR is the most frequently used technique for quantifying viral particles from samples. Despite its advantages, this technique does present some challenges, such as the need for a reference standard and a high degree of sensitivity as well as overestimation of infectivity where only the target sequence is measured and not the whole viral infective particle (Haramoto *et al.*, 2018b; Kralik and Ricchi, 2017; Hamza and Bibby, 2019). In recent years, digital PCR has attracted considerable attention globally due to its advantages over other methods. Digital PCR provides absolute quantification without the use of a standard curve and is less sensitive to complex matrices, such as environmental samples (Kishida *et al.*, 2014; Tiwari *et al.*, 2022; Sedji *et al.*, 2018).

The distribution of waterborne pathogenic viruses in/of major watercourses is currently unknown due to the lack of widespread monitoring if any. Due to the various types of viral pathogens, such as hepatitis A, rotavirus, adenovirus and norovirus, that are associated with waterborne transmission, their detection has not been simple. This is due to the lack of cell culture systems that are robust and sensitive enough to accommodate routine analysis (Nieuwenhuijse and Koopmans, 2017). Methods have not yet been developed to validate the detection of pathogenic virus prevalence and presence in river water. Molecular-based techniques have made major strides toward the detection and identification of viruses in the environment. Enteric viruses are still poorly understood in freshwater environments, despite advancements in viral detection techniques. The existing technologies available to recover and concentrate viruses from aqueous environments are inefficient, time-consuming, and expensive, making virus surveillance in freshwaters a hard task (Hayes *et al.*, 2023).

The transmission and stability of a virus is influenced by many environmental factors. Abiotic factors known to affect virus survival include temperature, climate, pH, UV light and water quality. Physiochemical parameters of river water are important to determine water quality. These parameters may also impact virus stability in a riverine environment such as

conductivity, turbidity, total suspended solids, total dissolved solids, nitrate, heavy metals and phosphate.

This chapter focuses on the adaptation of a method to concentrate, purify and detect waterborne enteric viruses in a riverine environment and assess their possibility as indicators for faecal contamination and compared to conventional indicators currently used. It also investigates the effects of abiotic factors on virus survival if any.

3.2 Methodology

3.2.1 Description of the study site

The Msunduzi River is located in Pietermaritzburg (PMB) in Eastern South Africa (SA), the provincial capital of KwaZulu Natal (KZN). It is more commonly known as the Duzi River and is a tributary of the Mngeni River, joining it just between Nagle Dam and Inanda Dam, thereafter flowing out into the Indian Ocean upon reaching Durban. Pietermaritzburg is a mixture of industrial, formal as well and informal suburbs and informal housing. The Duzi River passes through the centre of PMB in a narrow channel stretching 115 km long and up to 70 m wide. A portion of the river enters a canalised reach where it has been dammed by weirs allowing for canoeing and rowing practice, a stretch of river locally known as Camp's Drift. This part of the river was canalised to improve drainage capacity and prevent flooding which occurred previously in 1995. It also made land available for industry without the danger of regular flooding.

The Duzi River is home to many canoe races such as the Dusi Canoe Marathon, 50-Miler, Dash & Crash, Ngwenya Classic, Campbells to Duzi Bridge and Non-stop Dusi Marathon. The Dusi Canoe Marathon stretches approximately 120 km and is broken up into three stages: a 42 km, 46 km and 36 km stretch, respectively. The entire Duzi course is regulated by Henley Dam which releases water for canoe races depending on water availability at the time. However, the river itself is of poor water quality due to illegal dumping and sewers overflowing near the river during heavy rainfalls as seen in Figure 2.

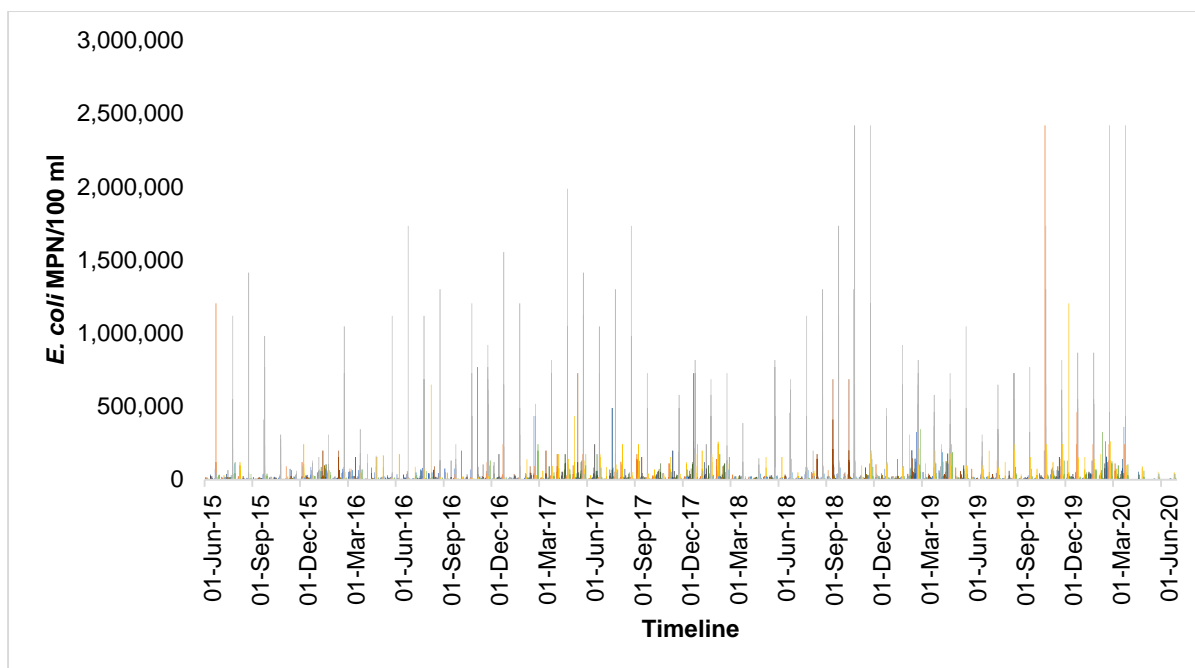


Figure 6. Five-year monitoring of *E. coli* results for the Msunduzi River and its tributaries (18 sites) showing many incidents of severe faecal contamination (>50 000 MPN/100 ml as referred to in Table 2) in this measure during the period.

The decline in the quality of the river is also due to the increase in urbanisation and informal settlements. It poses health concerns to canoeists such as diarrhoea, ear and eye infections as well as sepsis in open wounds and cuts. Duzi-Umgenei Conservation Trust (DUCT) is a non-government organisation set up to co-ordinate projects to enhance the quality of water, including conserving areas or regions surrounding the rivers. It has established guidelines for risk associated with canoeists when utilising the Duzi River for recreational purposes, as stipulated in Table 2 below.

Table 3. Risk to canoeists associated with *E. coli* levels present in recreational waters

| <i>E. coli</i> MPN/100 ml | Water quality assessment | Assessment of <u>probable</u> risk to <u>canoeists</u> |
|------------------------------|--|---|
| 0 – 130 | Considered acceptable for full contact recreation – excellent/likely natural catchment | Extremely low risk |
| 130 – 1 000 | Good – within the range expected for adequate catchment conditions, but significant/increased risk of illness if used for full contact recreation | Very Low risk |
| 1 001 – 4 000 | Fair - resource condition acceptable, natural sources are likely the source of <i>E. coli</i> , increasing the possibility of human faecal/sewage contamination. Unacceptable for swimming | Low, but increasing risk |

| | | |
|-----------------|---|---|
| 4 001 - 10 000 | Degraded - Significant faecal input or some sewage contamination is indicated. Other water quality parameters are likely to be poor | Moderate risk (approx. 1 in 10 may get sick) |
| 10 001 - 25 000 | Poor - sewage contamination present | Moderately high canoeing risk (approx. 1 in 5 may get sick) – some likelihood of illness, especially in white water |
| 25 001 - 50 000 | Very poor - significant environmental consequences | High risk (approx. 1 in 3 may get sick) – consider not canoeing or paddling on flat water only, with no risk of falling out |
| >50 000 | Severely contaminated | Very high canoeing risk – (approx. 1 in 2 paddlers may get sick) |

The Duzi River is joined by multiple small tributaries before picking up the effluent from the Darvill Wastewater Treatment Plant (WWTP). Darvill WWTP is the main WWTP for the Msunduzi Municipality treating industrial wastewater and raw municipal wastewater. There are large areas of PMB which used to be wetlands and floodplains. This capital has a population of over 300 000 people with access to waterborne sanitation, the size of the Msunduzi catchment being approximately 875 km².

3.2.2 Site selection and sampling

Sampling sites were selected from the start of the Msunduzi River surrounding the central area of the city of Pietermaritzburg, detailed in Table 4 and Figures 7 and 8. Further information can be found in Appendix B. This selection was based on historical *E. coli* contamination data, known river use for recreation and cultural activities and potential contributing factors to virus prevalence at each site, considering the little enteric virus research carried out on this stretch of the river. Samples were also selected from above and below a small impoundment called Henley Dam above the city from which water is released for the Dusi Canoe Marathon which is held annually. Although the upper Msunduzi catchment above Henley Dam, surrounding the Vulindlela area, has a significant population, it is far more rural in characteristics compared to the catchment below the dam. The impoundment itself also allows for potential risk assessment of the impact of impoundment on virus recovery.

Table 4. Sampling site description and co-ordinates along the Msunduzi River

| Site | Sample Description | Activities |
|-------------|--------------------------------------|---|
| S1 | Duzi Henley weir | 150000 population, pit latrines, mostly residential, field agriculture, overgrazed land, Vulindlela scheme water supply, forestry |
| S2 | Duzi Henley outflow | |
| S3 | Msunduzi at Caluza bridge | Sewer line, Mvubukazi, sewer coming through manholes |
| S4 | Msunduzi at Edendale weir | Ashdown stream, sewer crossing, manholes, sewer network, KwaPata settlement, Edendale hospital |
| S5 | Msunduzi above Dorpspruit confluence | Downstream of Camp's Drift, PMB CBD inputs, stormwater drain, old hospital, Foxhill spruit tributary, formerly sewered |
| S6 | Msunduzi above refuse dump | Dorpspruit and Ash Road squatter settlement, high-intensity housing, formally sewered, increase in population, electricity theft affecting sanitation |

For virus assessment, grab samples of 500 ml were taken fortnightly at each site in bacteriological bottles containing sodium thiosulphate. Samples were transported to the laboratory and stored at 4°C until processing within 48 hours of sampling. Sampling was carried out from March 2021 to September 2021 following the sampling protocol outlined in ISO 9001. A total of 72 samples were collected along the river, as per Table 4 and Appendix B (Table 12), and analysed. Samples were processed for the detection of enteric viruses in accordance with ISO 15216-1:2017 with the necessary modifications for digital PCR described in the methods below.

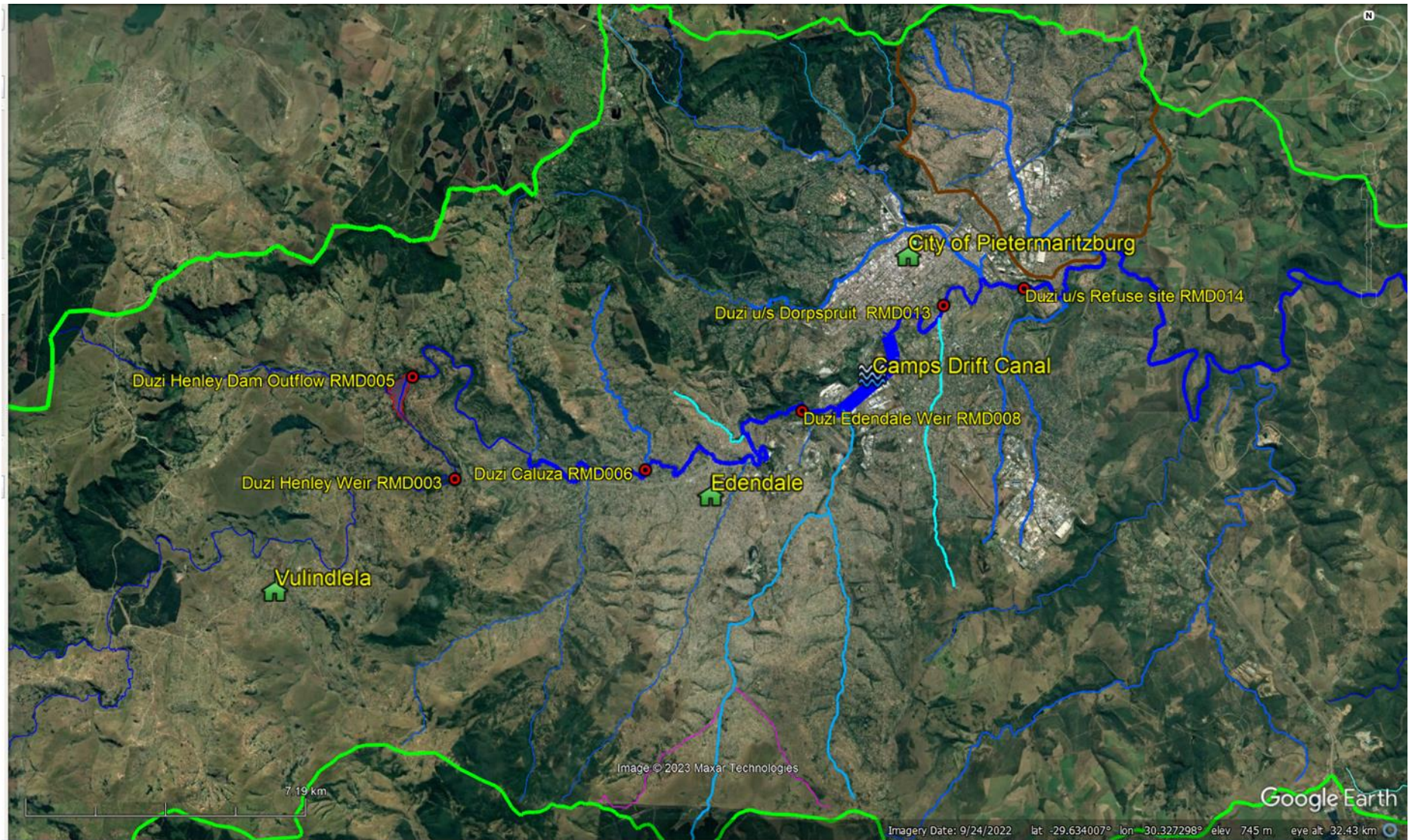


Figure 8. Study sample sites (6) on the Msunduzi River in the Pietermaritzburg area

3.2.3 Virus filtration and concentration

Reagents were prepared beforehand (refer to Appendix A). Samples were filtered with a peristaltic pump (Masterflex) using a 50 000 MWCO PES Vivaflow 200 cassette (Sartorius) with 0.2 µm pore size to remove bacteria. The unfiltered samples were first homogenised by inverting them several times and a 40 ml aliquot was decanted into a sterile 50 ml centrifuge tube. This tube was stored at -20°C for re-analysis if required. The remaining unfiltered samples were decanted into sterile bottles. The filtration system was installed and the filtration speed increased gradually until 2 bar pressure was obtained. Approximately 20 ml volume of sample was collected in a sterile 50 ml centrifuge tube.

A volume of 10 ml PEG 50% was added to the 20 ml filtered sample. The sample was incubated with constant reciprocal shaking at 70 rpm at 5±3°C for 60±5 minutes or overnight as viruses are stable in PEG solution and can be kept overnight with minimal loss in titres. Longer incubation may also enhance recovery.

After incubation, the sample was centrifuged at 11 000 x g for 30±5 minutes at 5±3°C. The supernatant was discarded. The pellet was air dried and either stored for 12 hours at 5±3°C or at -20°C for longer periods until further processing.

The pellet was resuspended in 1000±20 µl PBS 1X which was preheated at approximately 56°C. The tube was vortexed to homogenise the suspension. A volume of 1000±20 µl chloroform/butanol 1:1 solution was then added to the suspension and vortexed for 15 to 30 seconds. The sample was then incubated at room temperature for 5±0.5 minutes. After incubation, the sample was then centrifuged at 13 500 x g for 15±1 minute at 5±3°C. The aqueous or upper phase was then transferred into a sterile 15 ml centrifuge tube by pipetting the surface of the intermediate layer.

The aqueous phase was then stored for a maximum of 12 hours at 5±3°C or less than -20°C for long-term storage until nucleic acid extraction could be performed. The protocol was

adapted from ISO 15216:2017 with slight modifications (Le Guyader *et al.*, 2009; Baert *et al.*, 2011).

3.2.4 Extraction of nucleic acids

Nucleic acid extraction was performed using the MagMAX Total Nucleic Acid Isolation Kit (AM 1840) from LTC Technologies with the MagMAX express magnetic particle processor. A volume of 235 µl of Lysis Binding Solution was added to a bead tube with 175 µl of aqueous phase of the sample. The bead beating was done for 15 minutes as per the manufacturer's instructions. The beads were pelleted and 115 µl of the sample was transferred to a well of the MagMAX processing plate. A volume of 65 µl of 100% isopropanol was added to each well using a multichannel pipette and mixed for 1 minute. Reagents were added to the processing plate (refer to Appendix D) as per the manufacturer's instructions and placed in the MagMAX express and program AM 1840-V2 was used for extraction. Samples were eluted into 50 µl of elution buffer and stored at -80°C until digital PCR was performed using the primers and probes listed in Table 5.

The VetMAX Xeno Internal Positive Control (IPC) RNA (A29761) and DNA (A29762) together with the VetMAX Xeno IPC VIC Assay (A29767) (Life Technologies) were used as a positive control with each batch of samples to determine the extraction efficiency of the nucleic acid isolation kit and was carried out as per the manufacturer's instructions. The extraction efficiency was measured to monitor the extraction method used, magnetic extraction and bead beating. This may vary depending on the extraction method employed. The nucleic acid extraction efficiency of the method was calculated using the following equation and was deemed an acceptable efficiency as per ISO 15216:

$$\text{Extraction Efficiency (\%)} = \frac{\text{Total DNA/RNA copies recovered}}{\text{Total DNA/RNA copies spiked}} \times 100$$

3.2.5 Digital PCR

The QuantStudio 3D digital PCR system (Life Technologies) was used for this study. The primers and probes specific to the selected enteric viruses are listed in Table 5. Hepatitis A, rotavirus and norovirus GI and GII were reverse transcribed to produce cDNA templates using the 1 Step Fast Virus Master Mix (4444432) (Life Technologies) before digital PCR could be performed. Reaction volumes were 14.5 µl per chip comprising of 2 µl sample, 7.25 µl master mix, 0.725 µl assay (forward primer, reverse primer and probe) and 4.525 µl water. Amplification was performed according to the conditions listed in Table 6. Results were then analysed using the Analysis Suite Software (Life Technologies) designed for the QuantStudio 3D instrument.

Table 5. Primers and probes used for the detection of waterborne enteric viruses along the Msunduzi River using digital PCR

| Target | Primer or probe | Sequence 5' to 3' | Reference |
|--------------------------|-----------------|--|-----------------------------------|
| Rotavirus A | Forward | ACCCTCTATGAGCACAATAGTTA | (Joshi <i>et al.</i> , 2019) |
| | Reverse | TCACATAACGCCCTATAGCC | |
| | Probe | VIC-AGCTAACACTGTCAAAAACCTAA-MGBNFQ | |
| Adenovirus all serotypes | Forward | GCCCCAGTGGTCTTACATGCACATC | (Heim <i>et al.</i> , 2003) |
| | Reverse | GCCACGGTGGGGTTTCTAAACTT | |
| | Probe | FAM-TGCACCAGACCCGGGCTCAGGTA CTCCGA-TAMRA | |
| Adenovirus 40 and 41 | Forward | AACTTTCTCTCTTAATAGACGCC | (Ko <i>et al.</i> , 2005) |
| | Reverse | AGGGGGCTAGAAAACAAAA | |
| | Probe | FAM-CTGACACGGGCACTCTTCGC-TAMRA | |
| Hepatitis A | Forward | TCACCGCCGTTTGCCTAG | (Costafreda <i>et al.</i> , 2006) |
| | Reverse | GGAGAGCCCTGGAAGAAAG | |
| | Probe | FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ | |
| Norovirus GI | | Pre-designed assay | Thermofisher Scientific |
| Norovirus GII | | Pre-designed assay | Thermofisher Scientific |

Table 6. Amplification protocol used for digital PCR

| Stage 1 | PCR Protocol | | | | Cover Temp | Rxn Vol. |
|---------|--------------|---------|---------|--------|------------|-----------------|
| | Stage 2 | | Stage 3 | | | |
| 96.0°C | 60.0°C | 98.0°C | 60.0°C | 10.0°C | 70.0°C | 14.5 µl/chip |
| 0:10:00 | 0:02:00 | 0:00:30 | 0:02:00 | ∞ | | |
| 1x | 39x | | 1x | | | |

Plasmid controls were synthesized by GeneArt (Life Technologies) for each virus type and run with each batch of samples to ensure accurate results (refer to Appendix C). A minimum recovery rate of 1% of IPC was obtained for all the samples analysed thus validating the results according to ISO 15216-1:2019 criteria. The reaction mix was loaded onto the chip using the QuantStudio™ 3D Digital PCR Chip Loader. Each chip was uniquely tagged with a barcode and batch number. Once the chips were loaded, they were amplified on a dual flat block thermal cycler and thereafter the target concentration was read using the QuantStudio® 3D Digital PCR instrument. The process followed the workflow displayed in Figure 9 below.

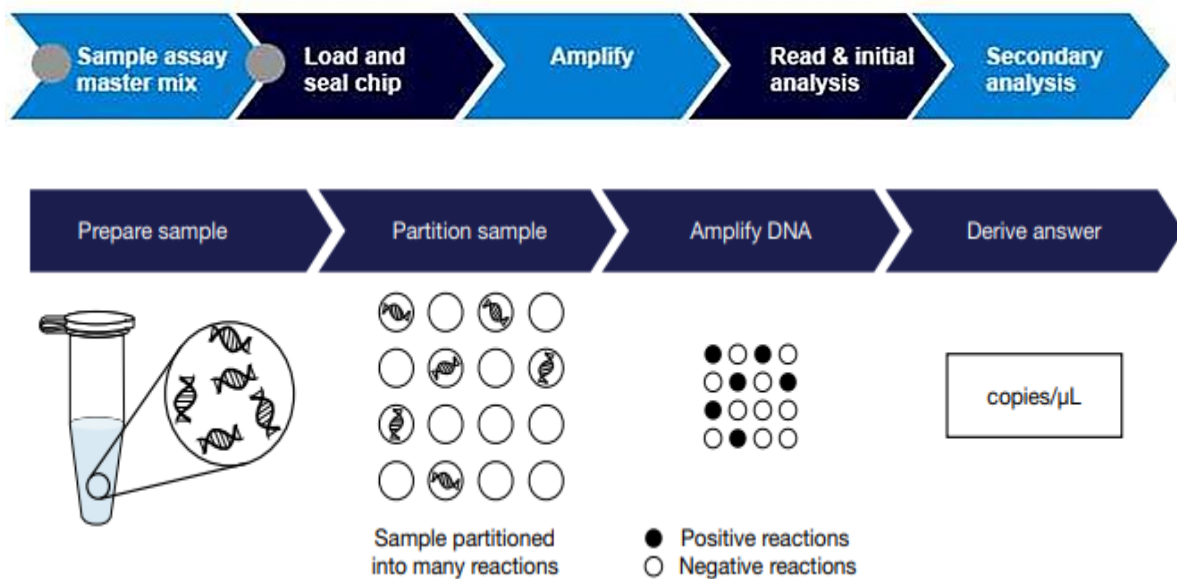


Figure 9. Digital PCR workflow adapted from ThermoFisher Scientific (Life Technologies).

3.2.6 Physiochemical characterisation

All sampling for physiochemical characterisation was undertaken according to ISO 9001 certified standards. Various analytical methods were performed according to Standard Methods for the Examination of Water and Wastewater, 24th Edition, to determine the physiochemical characteristics that may affect virus presence and survival. These include pH and temperature, turbidity, Biochemical Oxygen Demand (BOD), Total Organic Carbon (TOC), suspended solids, Chemical Oxygen Demand (COD), conductivity, alkalinity, ammonia, total phosphate and orthophosphate and rainfall. These methods are further explained in Appendix E.

3.2.7 Statistical analysis

A correlation matrix was done for each sample site in Microsoft Excel 2016 to understand the potential relationship between enteric viruses and abiotic and physiochemical parameters. The Pearson's correlation coefficient was used to describe linear relationships between these variables. Generally, $r=1$ denotes a strong positive direct correlation, $r=-1$ indicates a strong negative direct correlation and $r=0$ indicates no correlation. Refer to Appendix F for analytical data. The correlations found were interpreted according to Table 7 below.

Table 7. Pearson's correlation coefficient result interpretation

| Correlation | Interpretation |
|---------------------------|---|
| 0.9 to 1 (-0.9 to -1) | Very strong positive (negative) correlation |
| 0.7 to 0.9 (-0.7 to -0.9) | Strong positive (negative) correlation |
| 0.5 to 0.7 (-0.5 to -0.7) | Moderate positive (negative) correlation |
| 0.3 to 0.5 (-0.3 to -0.5) | Poor positive (negative) correlation |
| 0 to 0.3 (0 to -0.3) | Negligible correlation |

ANOVA was also carried out using GraphPad Prism 8 statistical software to determine if there were any significant differences between enteric viruses and environmental influences. Differences were considered significant at $p \leq 0.05$.

3.2.8 Other abiotic monitoring

Other sample analysis data were also available for the study sample points and were also utilised in statistical assessment. These included aluminum (Al), calcium (Ca), Potassium (K), fluorine (F), magnesium (Mg), sodium (Na), silicon (Si), sulphate, nitrate, iron (Fe) and manganese (Mn).

3.3 Results and Discussion

3.3.1 Quantification of enteric viruses from various sampling points in the Msunduzi River

The complexity of the matrix makes it difficult to quantify viruses in surface waters or other environmental samples. The concentration method used for isolation of the various viruses mentioned, included both ultrafiltration and PEG precipitation. Ultrafiltration allows for virus concentration from large volumes into smaller, more workable volumes for downstream processing (Goodrich *et al.*, 2020). Furthermore, PEG's strong viral precipitation capacity at potential ionic strength, neutral pH, and non-destructiveness to viruses make it an effective secondary concentration approach for the detection of enteric viruses (Ruhanya, 2016). After ultrafiltration using a 50 000 MWCO PES membrane, an ultracentrifugation step was needed to form a viral pellet. However, this step has disadvantages, such as the risk of viral disruption due to high centrifuge speeds causing shear. Therefore, to avoid ultracentrifugation and its disadvantages, a more efficient and cheaper alternative was used viz. PEG precipitation (Colombet *et al.*, 2007). The digital PCR method employed was sensitive enough to detect as little as 1 copy per μl of virus in a water sample and specific enough to differentiate between enteric viruses present in a single sample (Zhou *et al.*, 2021). The effectiveness of concentration methods also depends on the virus properties be it their surface charge or size (Forés *et al.*, 2022). Some studies have found ultrafiltration gave better virus recoveries as compared to PEG precipitation for recovery of some virus particles (Butot *et al.*, 2007). However, each method has its limitations.

In this study, filters were fouled quickly due to the turbidity present in river water samples. Ultrafiltration allows for multiple pathogens to be concentrated simultaneously and has achieved good recoveries (Forés *et al.*, 2021; Ye *et al.*, 2016).

Adenovirus both pathogenic and non-pathogenic, HAV, RVA, NoVGI and NoVGII were detected in all water samples analysed. Using optimised methods, all enteric viruses tested for were detected at least once in all six sampling sites indicating widespread possible sewage contamination. This is then highly suggestive that adenovirus, if quantification can be done accurately, could potentially be a good indicator for general human pathogenic virus presence. However, the routine monitoring for enteric viruses has not yet been included in water quality regulations (Hess *et al.*, 2021). Virus detection can be time consuming and labour intensive. Using a dPCR method, as described in this study, which is optimised for enteric viruses, can reduce cost and time.

The recovery efficiency of the method was calculated at 54% where ISO 15216 deems a recovery efficiency of >1% acceptable. Underestimating the true concentration by one to three orders of magnitude is common when using concentration processes and molecular approaches since virus recovery rates might be quite low (Mok and Hamilton, 2014; Petterson *et al.*, 2015). However, enhanced viral recoveries may be achieved by a further investigation into different concentration and extraction methods. An alternative process control such as mengovirus, also recommended in ISO 15216, can be used when testing the recovery efficiency of the method which may give a more accurate result as compared to the IPC used which is designed for animal health testing.

3.3.2 Detection of different enteric virus types in the Msunduzi River

A total of 72 samples were collected from different sampling points as described in section 3.2.1 over a period of six months. The concentration of the viruses varied across different sampling sites as well as the sampling period. Enteric viruses may have entered the Msunduzi River through various pathways, such as sewage contaminated with human faeces, livestock-

waste and wildlife. However, the primary source of enteric viruses in water is the faeces produced by infected people (Pang *et al.*, 2019). When human enteric viruses are present, they can spread across large distances and for extended periods by using water as a medium. Therefore, by transferring human enteric viruses to the human population, contaminated surface water can present a serious health risk. Using contaminated water as a direct source for drinking, irrigation of crops, food processing and lastly, recreational activities are common examples of this (Hess *et al.*, 2021).

Human adenovirus was detected in 100% of samples, as seen in Figure 10 and 11, which is in agreement with another study that concluded the highest viral pollution with adenovirus was found in river water (Forés *et al.*, 2022). Human adenovirus is considered to be the most prevalent enteric virus in water being known worldwide as the leading cause of waterborne disease associated with recreational activities (Sinclair *et al.*, 2009). The lowest and highest overall concentrations for all serotypes of adenovirus and pathogenic types 40 and 41 were found in sample Site 2, immediately below the Henley impoundment, hence low detection is logical and Site 3 respectively. The lowest concentrations of all serotypes of adenovirus and pathogenic adenovirus were detected in the month of May (end of the wet season) and August (mid-winter) respectively. The highest concentrations for pathogenic and non-pathogenic types were detected in August and April respectively. In addition to the fact that the contribution of various environmental stressors differs between viral species and seasons, different viruses experience varying degrees of inactivation. In a study (Li *et al.*, 2023) in summer, on average, compared to enterovirus and rotavirus, the percentage of adenovirus and norovirus inactivation caused by sun and thermal stresses was significantly higher which is in agreement with the highest concentration of pathogenic adenovirus being detected in August in this study. The slower rate of chemical degradation and microbiological decay in low temperature conditions during the winter months may contribute to a higher concentration. Reduced exposure to sunlight during the winter months may potentially have an impact on concentrations because of lower solar intensity and fewer daylight hours (Lenaker *et al.*,

2024). Although enteric adenoviruses of serotypes 40 and 41 are a major cause of gastroenteritis globally, especially in developing nations, little is known about their presence in water sources. This is mostly because traditional cell culture is unable to detect them (Schijven *et al.*, 2019). The average concentration of pathogenic adenovirus remained stable throughout the sampling sites with an increase in site 3 whereas non-pathogenic adenovirus remained stable with an increase in site 6.

There was 3.4 mm of rainfall on the day of sampling when the highest concentration of pathogenic strains was detected. Rainfall may increase contaminated levels in surface water due to the occasional discharge from surface runoff (Opere *et al.*, 2020). Human AdV is commonly present in groundwater, surface water and raw sewage and has been detected in water that has met water quality standards.

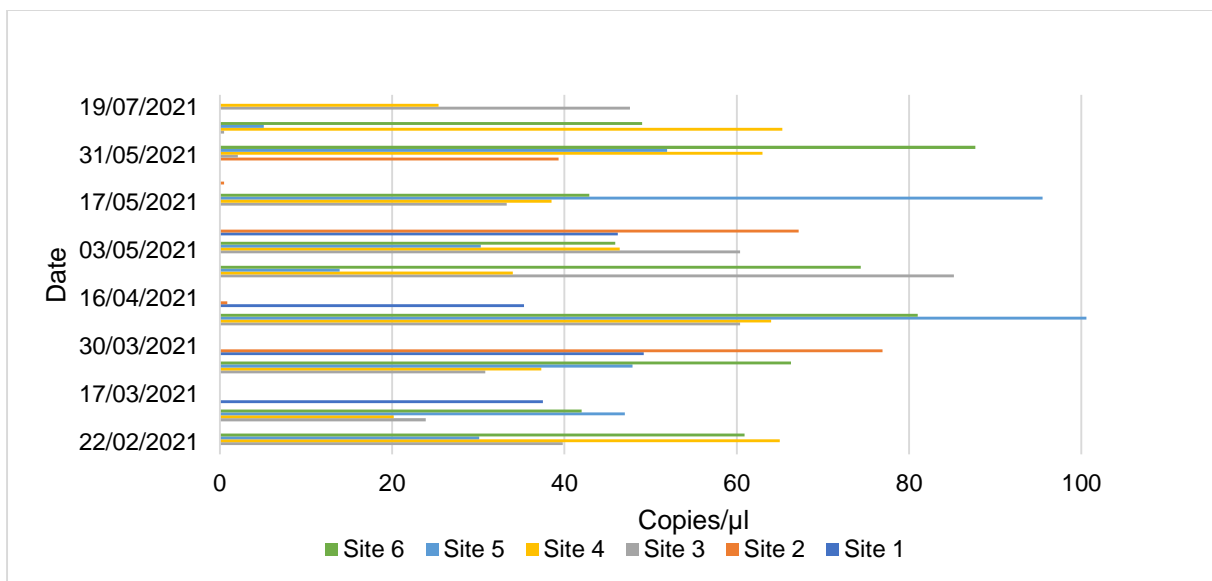


Figure 10. Adenovirus detection over 6 months in all study sites

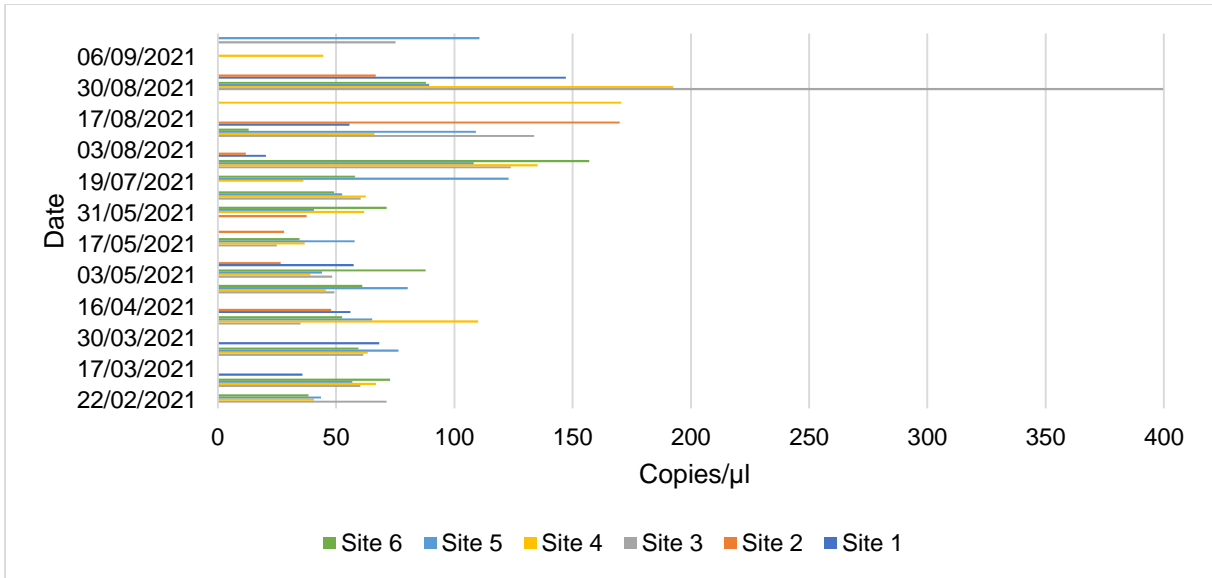


Figure 11. Adenovirus pathogenic strain detection over 6 months in all study sites

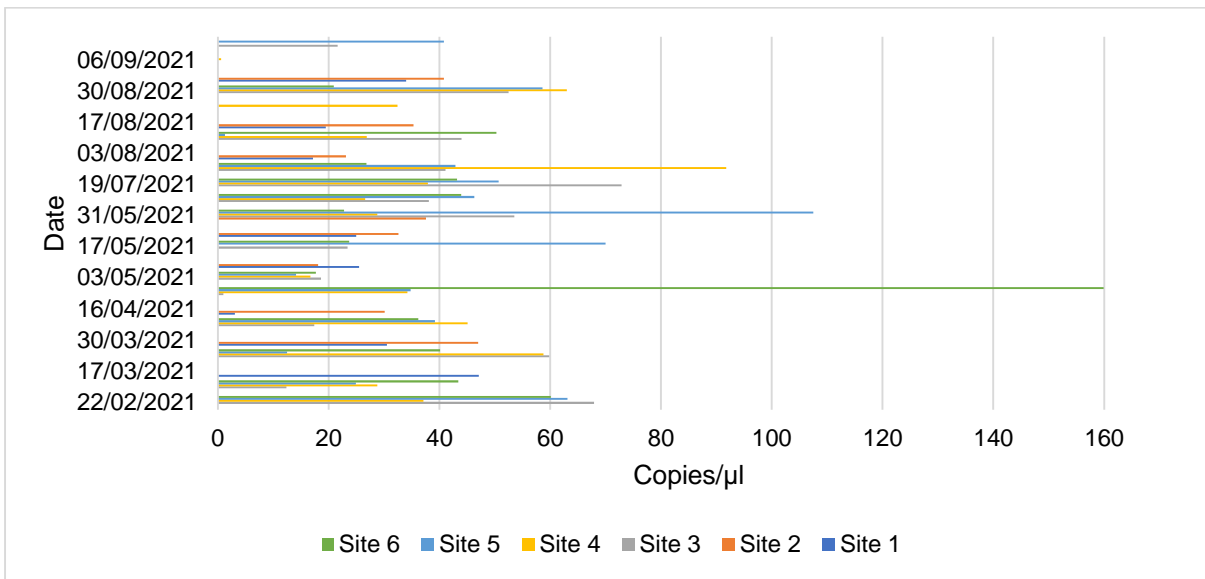


Figure 12. Hepatitis A detection over 6 months in all study sites

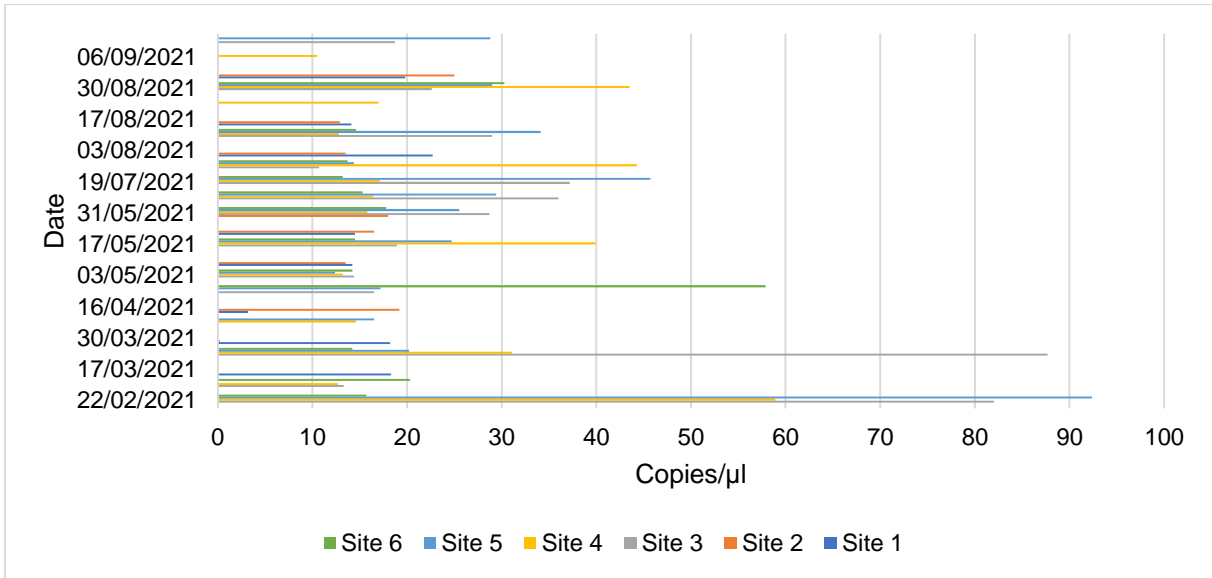


Figure 13. Rotavirus A detection over six months in all study sites

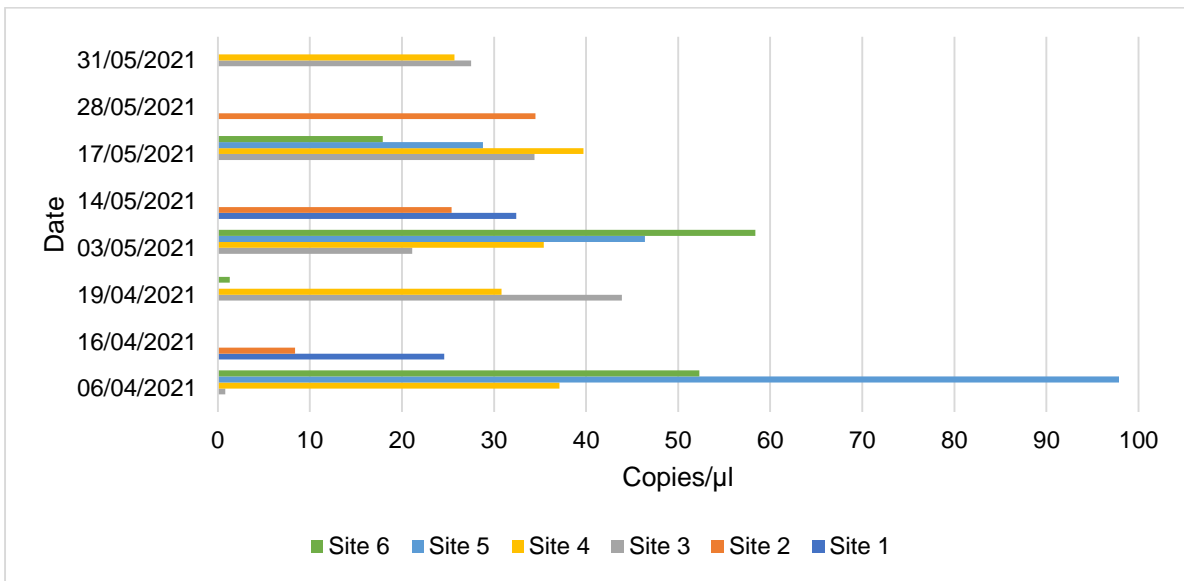


Figure 14. Norovirus GI detection over 2 months in all study sites

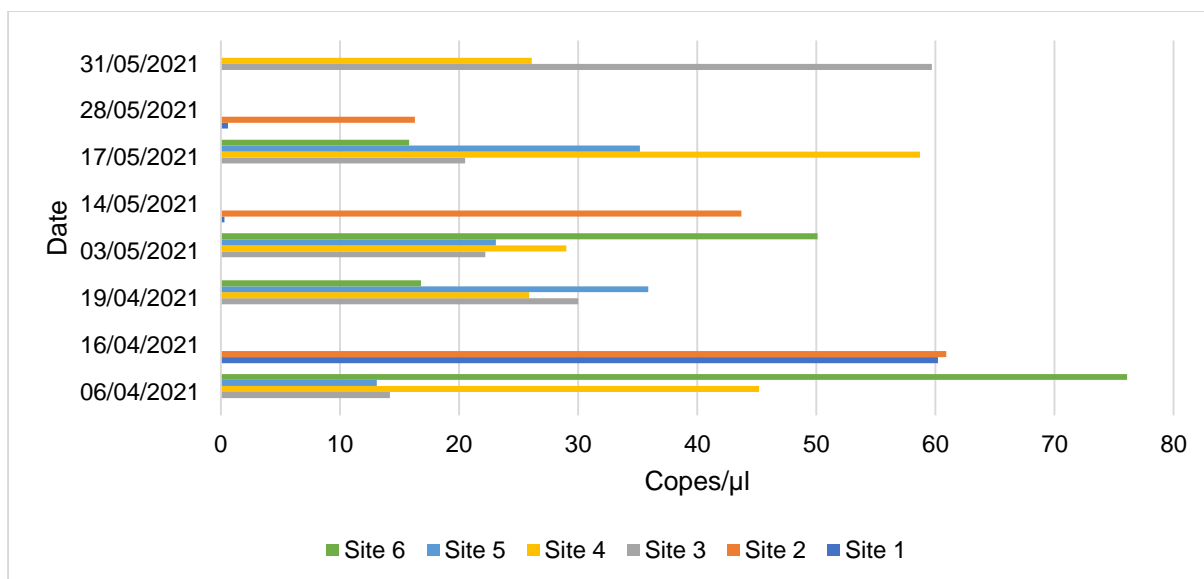


Figure 15. Norovirus GII detection over 2 months in all study sites

The lowest concentration of HAV was detected in Site 3 in September at 1000 copies/ml. The highest concentration of HAV was 159900 copies/ml in Site 6 coinciding with rainfall (3.00 mm) detected in April. The average concentration of HAV steadily increased along the Msunduzi River. Hepatitis A was detected in 98.6% of samples as seen in Figure 12.

The average concentration of rotavirus A remained stable, slightly increasing at sites 3 and 5 as seen in Figure 13. The lowest rotavirus A concentration was detected in March at Site 2. The highest concentration was found in March in sample site 5. This coincided with a report from the National Institute of Communicable Diseases (NICD). It states that rotavirus season in South Africa usually starts in May but can start as early as March (National Institute for Communicable Diseases, 2023). The season usually ends in September but can terminate as early as August and as late as October (Ismail *et al.*, 2016). In this dataset, rotavirus was detected from March through September. Seasonal patterns were not noticeable in this study as full seasons were not covered except for winter. Rotavirus A was detected in 98.6% of samples.

The lowest concentration of NoVGI was detected in April in Site 3 at 800 copies/ml and NoVGII in May in Site 1 at 300 copies/ml as seen in Figures 14 and 15. The highest concentrations of NoVGI (97900 copies/ml) and NoVGII (76100 copies/ml) were detected in the month of April in Site 5 and Site 6 respectively. A study in Singapore showed a higher prevalence of NoVGII as compared to GI in environmental waters (Goh *et al.*, 2019). Environmental surface waters at several areas also showed a comparatively high incidence of norovirus GII (Calgua *et al.*, 2013; Kishida *et al.*, 2012; Kiulia *et al.*, 2014). In this study, NoVGI showed a prevalence of 95.8% and NoVGII 91.7%, which does not correlate with these studies. However, the concentrations of NoV GII were higher in 4 out of 6 sites when compared with NoVGI concentrations. The concentration of NoVGI remained stable throughout the study with a slight increase in Site 5. The concentration of NoVGII started off lower in Site 1, as expected, and remained stable throughout the study. Previously, there was no available cell line for the norovirus infectivity test, therefore the detection of norovirus relied on molecular methods. However, a new three-dimensional (3D) cell culture method that uses human intestine enteroids (HIEs) produced from non-transformed stem cells has been used as a reliable and repeatable in vitro cultivation system for HuNoV (Wales *et al.*, 2024). Teunis *et al.*, (2008) demonstrated the low infectious dose for norovirus, where the 50% infectious dose, LD50 was equivalent to 1015 genomes copies or 18 viruses. Ngazoa *et al.* (2008) reported approximately 30% of norovirus genomes were detected in river water after 30 days of incubation at 25°C. This explains the high prevalence of norovirus in surface waters found in this study. Another study on viral persistence revealed that the reduction rate of nucleic acid of norovirus surrogates was significantly lower than the reduction rate of infectivity (Bae and Schwab, 2008). Nevertheless, extra precautions are needed when interpreting health risks brought by norovirus due to its low infectious dose.

In Site 1, NoVGI showed very strong positive correlation with both pathogenic ($r=1.000;p=0.0004$) and non-pathogenic adenovirus. A very strong positive correlation was also observed between non-pathogenic and pathogenic AdV ($r=1.000;p=<0.0001$). Very

strong negative correlations were observed between NoVGII and HAV ($r=1.000$) and RVA ($r=1.000$). Moderate positive correlations were observed between RVA and HAV ($r=0.631$) and NoVGI ($r=0.521$).

In Site 2, very strong negative correlations were seen between NoVGI and AdV ($r=-0.914$), differing from Site 1, and NoVGII ($r=-0.954$). However, a strong negative and positive correlation was observed between AdV and RVA ($r=-0.830$) and NoVGII ($r=0.751$) respectively. Moderate positive correlation was observed between AdV and HAV ($r=0.607$) and moderate negative correlation between RVA and NoVGII ($r=-0.618$).

In Site 3, very strong positive correlation was found between AdV and NoVGII ($r=0.950$) and moderate negative correlation between pathogenic AdV and NoVGII ($r=-0.668$). Strong positive and negative correlation was observed between NoVGII and HAV ($r=0.748$) and NoVGI and RVA ($r=-0.853$) respectively.

In Site 4, strong positive correlations were seen between NoVGII and HAV ($r=0.837$) and NoVGI ($r=0.819$). Moderate positive correlation was observed between NoVGI and HAV ($r=0.602$).

In Site 5, moderate positive correlations were seen between NoVGII and RVA ($r=0.540$) and NoVGI ($r=0.547$). A moderate negative correlation was observed between NoVGI and pathogenic AdV ($r=-0.690$) and a strong positive correlation between NoVGI and AdV ($r=0.817$).

Lastly in Site 6, moderate positive correlations were found between pathogenic and non-pathogenic AdV ($r=0.624$; $p=0.0171$) and AdV and NoVGI ($r=0.501$). Strong positive correlations were found between NoVGII and RVA ($r=0.834$) and NoVGI ($r=0.861$). A strong negative correlation could be seen between HAV and NoVGI ($r=-0.750$).

Significant associations were only observed in Site 1 and 6, between pathogenic and non-pathogenic adenovirus and NoVGI and pathogenic AdV. Overall, based on the results, it can be deduced that adenovirus is a good predictor/indicator of other enteric viruses present in

the Msunduzi River. No seasonal pattern was observed overall for the enteric viruses tested which concurs with other studies which observed RVA, HAV, NoVGI and NoVGII among others (Girón-Guzmán *et al.*, 2024; Masachessi *et al.*, 2021; Janahi *et al.*, 2020). In a study conducted by (Prez *et al.*, 2015), there was no significant seasonal change that permanently contributed to the contamination observed in the study location.

3.3.3 Correlation of physiochemical factors to the concentration of viruses across the Msunduzi River

The relationship between the occurrence of enteric viruses and the physiochemical properties of water quality were assessed from water samples collected from the Msunduzi River in Pietermaritzburg, South Africa. In this study, the major physiochemical parameters (turbidity, COD, BOD, TOC, pH, temperature, rainfall, and suspended solids) were monitored across the sampling period and were correlated with the viral abundance. Additional data was available for other water quality parameters and was used to populate the correlation matrix. The correlation matrix for each site can be found in Appendix F.

In water, the forms of nitrogen of greatest interest are organic nitrogen, nitrite, nitrate and ammonia. All these forms of nitrogen, as well as nitrogen gas, are biochemically interconvertible and other components of the nitrogen cycle (i.e., ammonification, nitrification and denitrification). The probability of some relationship with ammonia was found to be statistically significant to AdV ($p=0.0266$), NoVGI ($p=0.0370$) and NoVGII ($p=0.0485$) in sample sites 3, 2 and 4 respectively as seen in Figures 17-A, 16-F and 18-B. Ammonia showed a very strong negative correlation with NoVGI ($r=-0.998$) and a strong positive correlation with NoVGII ($r=0.8809$) respectively. Quaternary ammonium is listed as one of the EPA's registered antimicrobial products effective against norovirus (feline calicivirus). This may explain the strong negative correlation with NoVGII. Ammonia is abundant in surface water and is an important faecal pollution indicator. During industrial processes, in sewage effluent and agricultural runoff, ammonia is emitted into surface water systems (Ding *et al.*, 2021). A study confirmed that as the concentration of ammonia decreased, the concentration of

pathogenic adenovirus increased. Adenovirus inactivation was proven to occur at a high pH and in the presence of ammonia (Magri *et al.*, 2015). In other cases, quaternary ammonia solutions were found to be ineffective against norovirus which may explain the positive correlation of ammonia and NoVGI (The California Department of Public Health, 2018).

Rainfall was shown to be statistically significant and showed a strong positive correlation when compared with RVA concentrations in both Site 3 ($p=0.0007$, $r=0.7951$) and Site 6 ($p=0.0394$, $r=0.7648$) as seen in Figures 17-D and 19-C. A moderate to strong positive correlation was seen in both sample points. A study in Kenya contradicted these results where adenovirus and enterovirus were not significantly associated with the wet or dry season (Opere *et al.*, 2020). In another study, rainfall was found to be positively but weakly correlated with microbial indicator presence in water (Goh *et al.*, 2019).

Suspended solids were statistically significant when compared with concentrations of AdV 40 and 41 in Site 3 ($p=0.0017$, Figure 17-A) and RVA in Site 6 ($p=0.0104$, Figure 19-B). Pathogenic adenovirus showed a positive correlation with $r=0.7586$. Viruses may become inaccessible based on the number of suspended solids available in a sample that they are adsorbed to. Other dissolved chemicals present in the sample can affect adsorption capacity. Physiochemical models have been suggested to account for the strong adsorption of viruses onto the surface of porous suspended solids (Petala *et al.*, 2021). Differences between turbidity and AdV 40 and 41 ($p=0.0115$, $r=0.8299$), as well as NoVGI ($p=0.0466$), were found to be statistically significant in sample Sites 3 (Figure 17-F) and 5 (Figure 18-F) respectively. Suspended solids have a direct effect on turbidity, as the amount of suspended particulate increases, so will the turbidity of that sample. Capsid proteins, viral envelope structure and particle size may affect enteric virus adsorption and desorption to particulate matter (Yang *et al.*, 2022). Virions attach to suspended material in the sample which protects them from inactivation allowing them to survive for extended periods in the environment (Boussetine *et al.*, 2020).

Temperature is the most evaluated environmental factor that affects virus survival. It has been acknowledged as one of the crucial physicochemical factors affecting a micro-organisms ability to survive in these conditions. Higher temperatures result in more rapid inactivation of viruses. It has been demonstrated that pathogen survival in aquatic environments is longer at lower temperatures i.e. 4°C and -20°C (Ibrahim *et al.*, 2019). Temperature, pH, turbidity and TOC have been associated with viral concentration where HAdV showed a significant correlation with high TOC which is in agreement with the current study (Prado *et al.*, 2019). Total organic carbon concentrations were found to be statistically significant with AdV in Site 1 ($p=0.0222$), Site 3 ($p=0.0018$), Site 5 ($p=0.0041$, $r=0.7143$) and NoVGI ($p=0.0267$, $r=0.9733$) and NoVGII ($p=0.0454$, $r=0.9546$) in Site 6. Moderate to strong positive correlation is shown in sample sites 5 and 6 respectively.

In sample sites 3 (Figure 17-E) and 5 (Figure 18-E) statistical significance was found between AdV ($p=0.0411$) and HAV ($p=0.0112$) and total phosphate concentrations respectively. High phosphorous levels can lead to eutrophication in rivers where high nutrient levels allow for excessive plant and algal growth. This can adversely affect water sports and recreational activities and cause the loss of both plant and animal life in these waters. Effluents and wastewater contain large concentrations of phosphorous which are discharged into watercourses. Therefore, the presence of phosphates in water indicates faecal pollution. Another source of phosphorous is agricultural land runoff. A study (Boehm *et al.*, 2019) confirmed the relationship between nutrients in water and the survival of waterborne viruses where the average decay rates of waterborne viruses in surface waters were documented to range from 0.07 to 0.9 per day. This confirms that heavy loads of nutrients in water that is contaminated by wastewater can override the stress caused by non-optimal conditions such as temperature, high salinity and toxic chemicals thereby prolonging the survival of microorganisms (Gholipour *et al.*, 2023).

Statistical significance was shown between aluminum and AdV ($p=0.0323$) in Site 1 as seen in Figure 16-A. This was also seen in Site 6 with concentrations of AdV ($p=0.0452$) and NoVGII ($p=0.0343$, $r=0.9657$) depicted in Figure 19-A.

Statistical significance was determined between pH and HAV in sample Site 1 ($p=0.0315$, $r=0.798$) and AdV 40 and 41 in Site 5 ($p=0.0429$). Correlation analysis in a recent study showed that pH, solids and temperature had the most influence on virus removal from WWTPs (Plaza-Garrido *et al.*, 2023). Physiochemical parameters determined to be statistically significant only in sample Site 2 include temperature ($p=0.0336$) and dissolved oxygen ($p=0.0463$, $r=0.739$) with AdV 40 and 41 and sulphate with NoVGII ($p=0.0375$, $r=0.998$). A study revealed that dissolved oxygen had the highest correlation with viral copies in terms of absorption ratio to suspended solids (Petala *et al.*, 2021). Viruses can persist suspended in environmental waters at both low and high temperatures for several days (Opere *et al.*, 2020). Biochemical oxygen demand was only found to be statistically significant with NoVGI in Site 4 where $p=0.0351$. Conductivity was determined to be statistically significant showing a positive correlation ($r=0.9612$) when compared with NovGI concentrations in Site 5 where $p=0.0296$ as seen in Figure 18-G.

Contrary to the results in this study, a study in Brisbane, Australia indicated that there was a poor correlation between physiochemical parameters and the presence of enteric viruses in both influent and effluent wastewater treatment samples (Sidhu *et al.*, 2018). This suggests that the use of physiochemical parameters to predict the presence of enteric viruses has its limitations (Opere *et al.*, 2020).

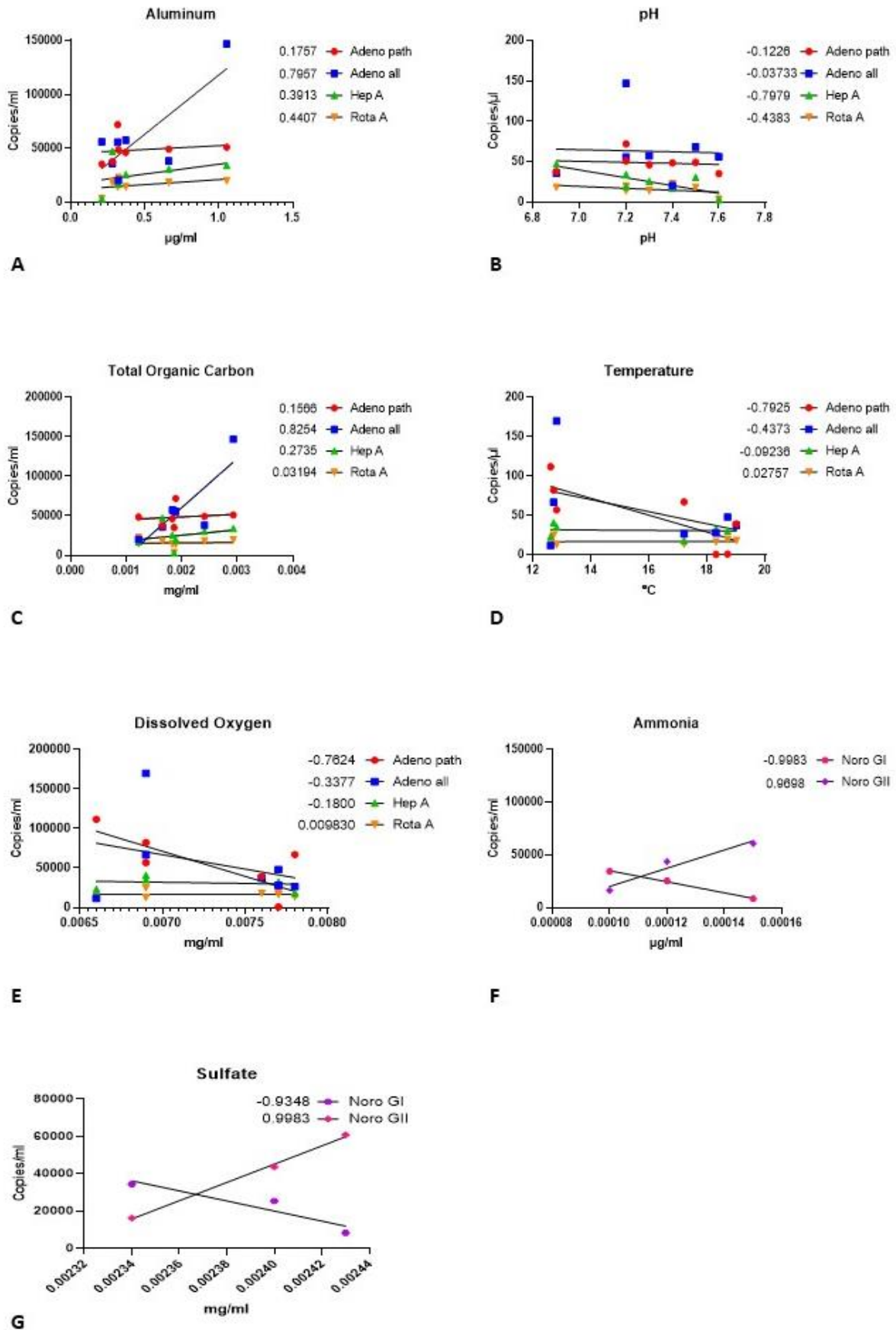


Figure 16. Significant associations between physiochemical parameters and enteric viruses in Site 1 (A-C) and Site 2 (D-G).

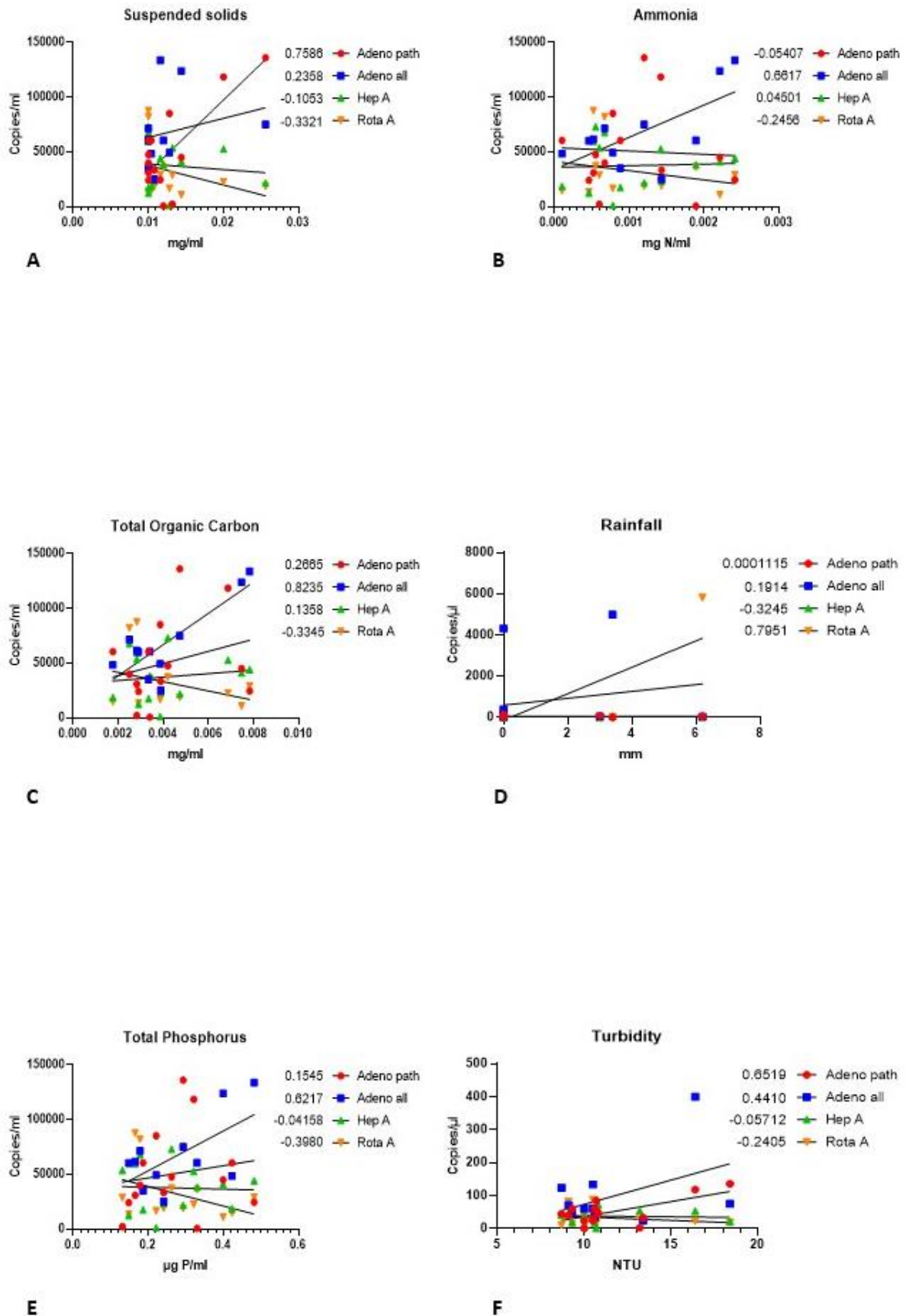


Figure 17. Significant associations between physiochemical parameters and enteric viruses in Site 3

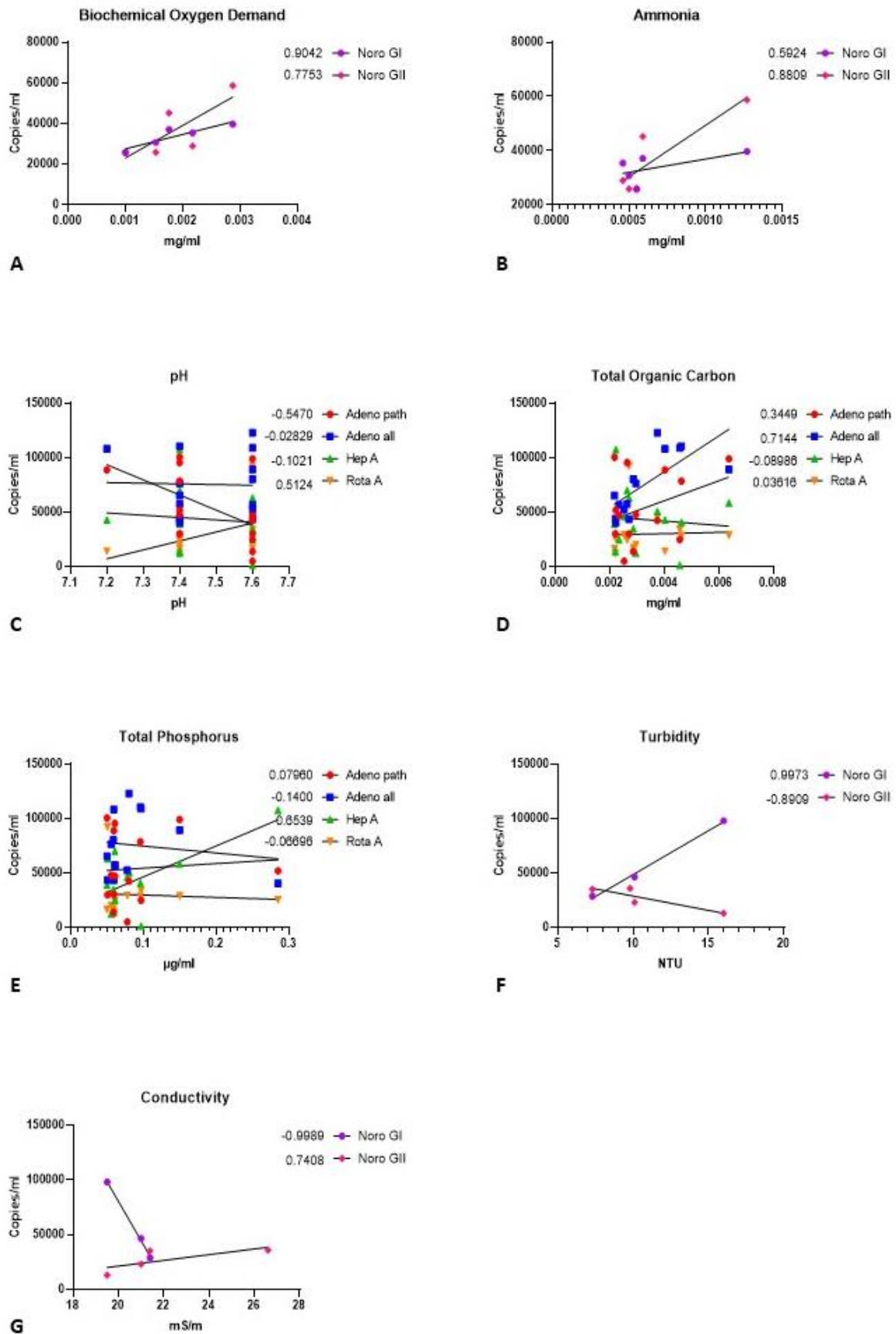


Figure 18. Significant associations between physiochemical parameters and enteric viruses in Site 4 (A-B) and Site 5 (C-G).

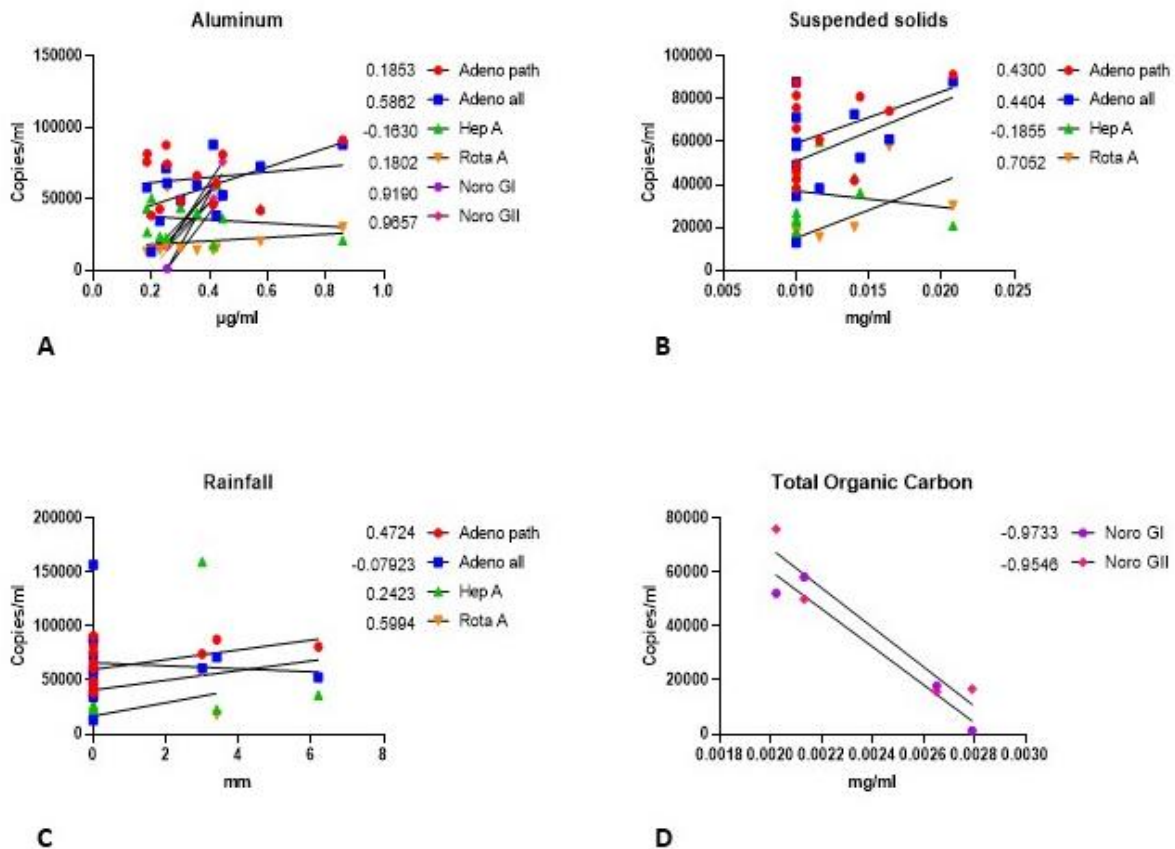


Figure 19. Significant associations between physiochemical parameters and enteric viruses in Site 6

3.4 Conclusion

The present study reports on the quantification of enteric viruses present in a riverine environment. The optimised method was sensitive and specific enough to detect each virus type tested in this study. The study also revealed the relationships between physiochemical parameters and the survival of waterborne enteric viruses which coincided with literature. Statistically significant associations were found between enteric viruses and pH, rainfall, TOC, Al, NH₃, total phosphorous, turbidity and suspended solids at more than one sample site. The dataset for norovirus testing should be revisited due to a smaller amount of samples being tested for as compared to the other types of viruses. By increasing the dataset, more accurate results and correlations can be determined for norovirus.

CHAPTER FOUR – ESTABLISHING RELATIONSHIPS BETWEEN ENTERIC MICROBIAL INDICATOR LOADS, ANTHROPOGENIC ACTIVITIES AND WATERBORNE PATHOGENIC ENTERIC VIRUSES

4.1 Introduction

Increasing urbanisation, industrialisation and agricultural activity have adversely affected the quality of surface water worldwide. Raw or poorly treated sewage leaks or discharges into water sources are the primary means by which enteric viruses infiltrate the environment. The use of contaminated water for drinking, cooking, or recreational purposes such as leisure and sports subsequently results in human exposure (Upfold *et al.*, 2021). In addition, the ageing wastewater and sewage infrastructure in South Africa contributes significantly to surface water pollution due to inadequate treatment before discharge. This can be confirmed in the Green Drop Report issued by The Department of Water and Sanitation where wastewater networks and treatment systems were found to be in a critical state in KZN (Department of Water and Sanitation, 2023). Traditionally, *E. coli* is used as an indicator organism to assess the microbial quality of water. Other commonly employed indicators include human enteric viruses which are also associated with faecal contamination (Osuolale and Okoh, 2017). However, there are limitations when using faecal bacteria alone as water quality indicators. Therefore, coliphages as alternative indicators were introduced to address the lack of correlation between faecal indicator bacteria and viral pathogens (McMinn *et al.*, 2018). Furthermore, because they are found in both human and animal faeces, coliphages and faecal indicator bacteria cannot be used to solely detect human faecal pollution. Additionally, the number of infectious viral particles required to cause infection is much lower than bacteria. Human-specific viral indicators can be used to provide quantitative evaluations of wastewater pollution, viral degradation, and virus transport in water. It has been reported that there is a weak correlation, or no correlation at all, between indicators and viral pathogens indicating disparate results in literature (Goh *et al.*, 2019). Waterborne enteric viruses can be introduced into the

environment through sewer overflows, agricultural runoff, urban runoff and wastewater discharge.

This chapter focuses on determining whether there is a relationship between enteric microbial indicators used to determine water quality and enteric viruses based on a case study. The Dusi Canoe Marathon is held annually on the Msunduzi River. The Dusi Canoe Marathon stretches approximately 120 km where the entire Dusi course is regulated by Henley Dam, which releases water for canoe races depending on water availability at the time. The effect of this marathon on enteric virus load in comparison to *E. coli* is assessed to determine risk and contamination levels of the water before and after the race. This chapter will also provide insight into possible sources of contamination along the river and whether one of the six enteric viruses tested for can be used as a possible viral indicator in river water.

4.2 Methodology

4.2.1 Sample collection and storage

Samples were collected fortnightly in 500 ml volumes from the upper Msunduzi River in Pietermaritzburg at specific sampling sites as described in Table 4 in bacteriological bottles containing sodium thiosulphate (JVL Laboratory, Engineering and General Supplies). Routine sampling took place between March 2021 and September 2021. Sampling for the Dusi marathon was carried out between February 2021 and April 2021 including pre and post-marathon sampling. Samples were collected weekly in the 3 weeks leading up to the marathon, during the 3-day marathon and weekly 3 weeks after the marathon in the same manner from various sampling sites. Samples were transported to the laboratory at 4°C and stored until sample processing, within 24 hours of sampling. Quality control bottles were also tested with each batch of samples taken to ensure accurate and consistent results proving the bottles were free from bacteriological and viral contamination serving as procedural blanks. The sampling protocol used was carried out following ISO 9001. A total of 72 routine samples were collected and analysed to determine enteric microbial load. However,

during the Dusi Marathon, around 100 samples from before, during and after the marathon were collected.

4.2.2 Enumeration of enteric microbial indicators in river samples

Approximately 100 ml of sample was used to enumerate *E. coli* using the Colilert 18 method (Idexx, USA). Samples were transported at 4°C to the laboratory and analysed upon arrival. Samples were diluted when higher levels of contamination were suspected, as required and results were reported as Most Probable Number (MPN) per 100 ml.

Samples were analysed for somatic coliphages using the double-layer agar technique according to ISO 10705-2 with a few modifications. The host strain used was *E. coli* ATCC 700609, a nalidixic acid-resistant mutant of *E. coli* ATCC 13706. The media composition for the Phage Bottom Agar (PBA) and Phage Top Agar (PTA) are listed in Appendix G. Results were recorded as Plaque Forming Units (PFU) per 10 ml.

4.2.3 Virus detection and enumeration in river samples

Samples were processed for the detection of enteric viruses according to ISO 15216-1:2017 with the necessary modifications made for digital PCR. Virus concentration, extraction, detection and enumeration were carried out on all samples as per the method stipulated in Chapter 3 for adenovirus, hepatitis A, rotavirus A, norovirus GI and norovirus GII using digital PCR.

4.2.4 Representation of enteric viruses detected in the Dusi Canoe Marathon

The virus sample detection during the canoe marathon was separated into pre, during and post-marathon. The GPS coordinates for each point sampled throughout the marathon and individual virus concentrations were used to create heat maps using Geographic Information System (GIS) software in this case Arc GIS 10.6. Statistical analysis was performed by way of a correlation matrix and linear regression using GraphPad Prism 8.0 software.

4.3 Results and discussion

4.3.1 Anthropogenic factors affecting the survival of enteric viruses

Sewage is one of the major sources via which enteric viruses enter the environment into source waters such as rivers, dams and streams (Upfold, Luke and Knox 2021). Using contaminated environmental waters may lead to viral disease outbreaks even where contamination levels may seem minimal. Therefore, a more comprehensive analysis of contamination and risks must include additional indicators, such as enteric viruses (Holcomb and Stewart, 2020).

All viruses investigated in this study were found to be prevalent in the Msunduzi River, one of the major rivers in PMB. In addition to serving as a source of fresh water for many rural and informal settlements in and around PMB, this river course is also the site of the world-famous Dusi Canoe Marathon race. The cause of faecal contamination in this river may be attributed to various human activities such as inadequately treated wastewater discharge, untreated effluent discharge from settlements, recreational activities and flooding.

Human and natural factors may also contribute environmental and climatic change. Human activities affecting climate change include releasing harmful gases into the atmosphere. These gases may be produced through the burning of fossil fuels, deforestation and in particular industrial activities (Uwishema *et al.*, 2023). The Msunduzi River is surrounded by various industries which dispose various effluents and chemicals directly into the river resulting in changes to the aquatic ecosystem. These changes may impact virus prevalence along the river.

The climate affects public health where climate change can influence the transmission of infectious diseases which is dependent on climatic and environmental changes. Reduced river flows brought on by droughts and poor rainfall lead to an increase in the concentration of waterborne viruses. On the other hand, when floodwaters become contaminated with human or animal waste, strong downpours and subsequent floods after droughts can prolong the

spread of diseases to people, leading to a rise in the faecal-oral transmission of diarrheal diseases (Uwishema *et al.*, 2023). This phenomenon may have occurred when a large volume of water was released from the Henley impoundment before the Dusi Canoe Marathon. This would have resuspended particulate matter along the Duzi River and viruses have been known to adsorb to the surfaces of suspended solids.

4.3.2 Impact of the Dusi Canoe Marathon on viral distribution

The direct monitoring of viral contamination in recreational waters is a rare occurrence. The highest average concentration of enteric virus found 3 weeks before and during the race was AdV 40 and 41 as seen in Figure 15 below. However, 3 weeks after the race, RVA was found to have the highest average concentration. The data obtained during the post-marathon sampling indicate the survival of enteric viruses in water. Not all viruses can survive for long periods in water. This could also be due to river flow where the viruses may have been transported downstream of the river. One study using meta-analysis determined the overall prevalence of adenovirus in water to be just over 59% and 49.5% in surface water (G. R. Takuissu *et al.*, 2023). Their findings suggested that water was one of the main routes of AdV transmission in both developed and developing countries. A study in Uganda modeled rotavirus concentrations in river water. It was determined that pathogen loading contributors were urban open defecation, treated effluent from WWTPs and unsafe disposal of faecal sludge. Urban areas with high population densities and a predominance of slum housing issues were hotspots for RV pollution (Okaali *et al.*, 2021). Hepatitis A may endure in a typical environment for months since it is stable in water with a low pH and moderate temperature (Ryu *et al.*, 2019). Viral populations within the environment may either remain stable or decline in the absence of host cells (Pinon and Vialette, 2019). Adenovirus types 2 and 41 were found to be stable in surface waters with a temperature of 10°C for 160 days and in some instances up to 301 days. At 19°C the virus concentration showed a 4log reduction after 301 days (Rigotto *et al.*, 2011). Norovirus GI concentration remained constant whereas NoV GII concentration decreased by 50%. It has been discovered that these viruses can survive for

weeks to months in groundwater, river water, mineral water, and tap water (Zhu *et al.*, 2020). According to a study on long-term persistence, norovirus and adenovirus genomes can both survive in water for up to 1277 and 1343 days, respectively (Kauppinen *et al.*, 2018).

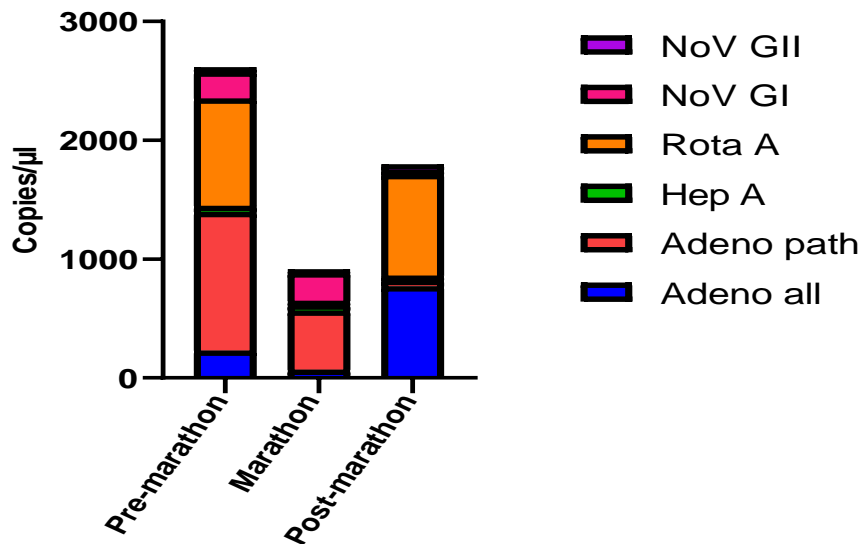


Figure 20. Average enteric virus concentrations detected before, during and after the Dusi Canoe Marathon, 2021.

To assess the impact of the Dusi Canoe Marathon on the prevalence of enteric viruses, sampling was done pre, during and post-marathon as seen in Figure 20. The concentrations of the enteric viruses are depicted in Figures 21 to 23. Before the marathon, enteric virus concentration was detected more closely upstream. There was an increase in enteric virus concentration between the end of Day 1 of the marathon and the start of Day 2 for AdV (111%), RVA (39%) and HAV (85%). Adenovirus 40 and 41 showed the most significant increase in concentration. This implies that human activities contribute to enteric virus concentrations in environmental waters. However, during the marathon, the relative concentration of viruses moved along the stretch of the river. From the data obtained, it can be determined that the canoe marathon did affect the distribution of viruses throughout the Msunduzi River, indicating that anthropogenic activities can introduce enteric viruses into environmental waters and affect their concentrations.

The distribution of HAV and RVA was found to be similar during the race. The lower levels of viruses detected during the race may be attributed to the dilution rate as water is released from the Henley impoundment each year before the race. This results in a higher flow rate and volume of water.

In one study, (Kraay *et al.*, 2018) the environmental role of rotavirus transmission was found to be affected by river flow velocity, temperature and the size of the water body determining the dilution rate. An increase in temperature increases the decay rate of RV. Pathogens may gather in slow-moving places near river edges in communities with flowing surface water sources.

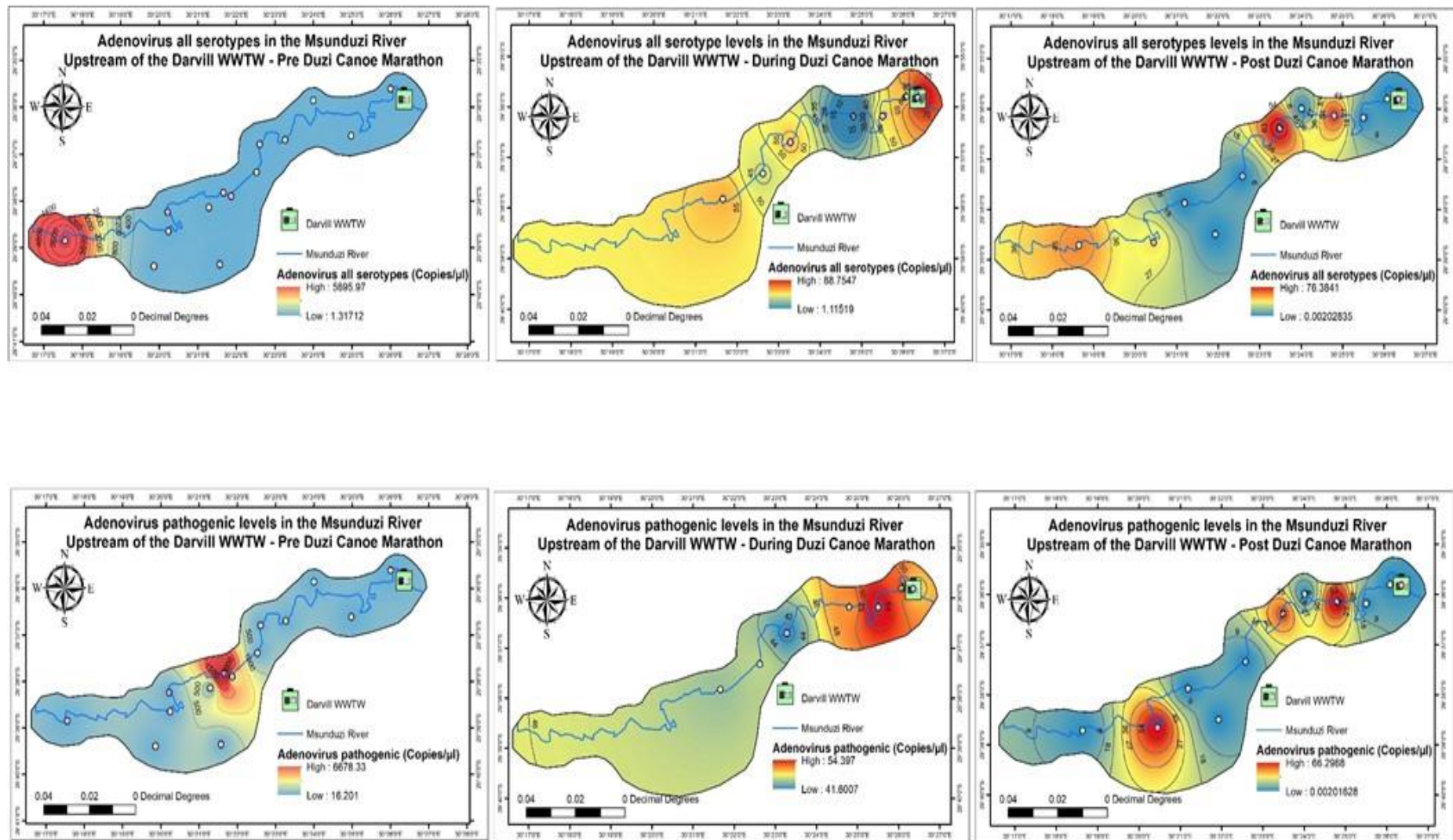


Figure 21. Adenovirus progression in the Msunduzi River during three time intervals for the Duzi Canoe Marathon 2021.

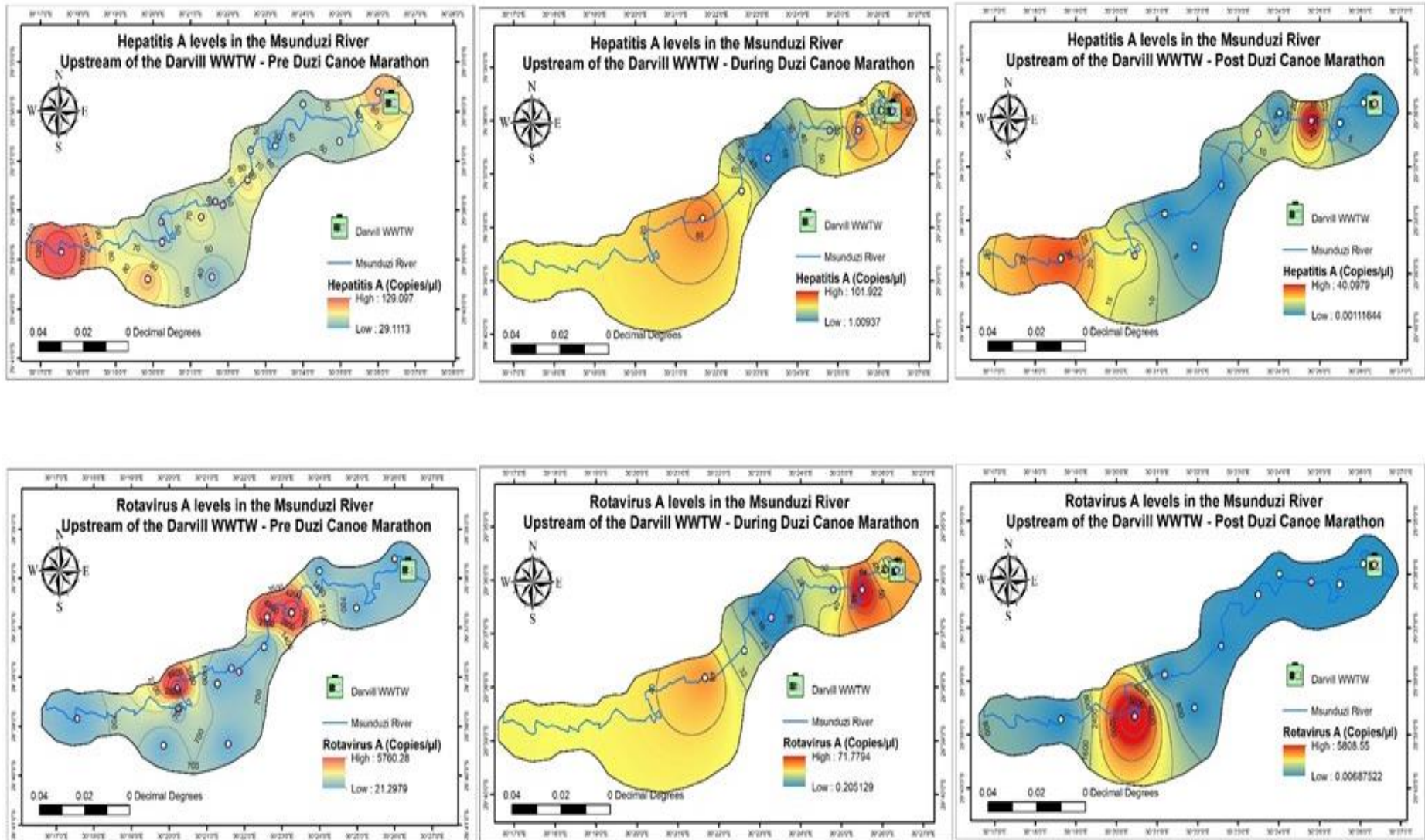


Figure 22. Hepatitis A and Rotavirus A progression in the Msunduzi River during three time intervals for the Duzi Canoe Marathon 2021.

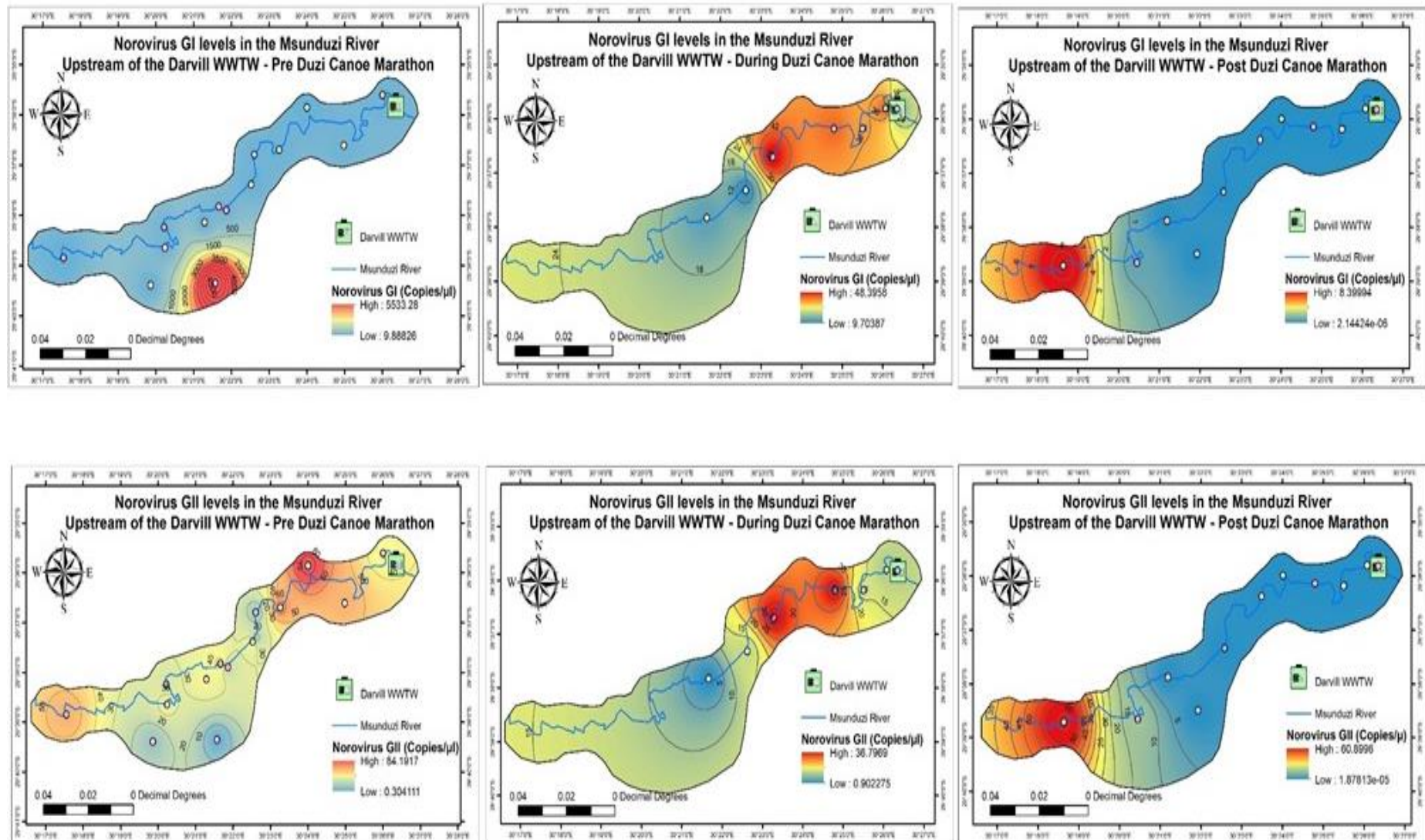


Figure 23. Norovirus GI and GII progression in the Msunduzi River during three time intervals for the Duzi Canoe Marathon 2021.

4.3.3 Relationship between enteric microbial load and enteric viruses

To understand the relationship between traditional faecal indicators and enteric viruses, a correlation matrix and linear regression using GraphPad Prism 8.0 software were performed. However, no correlation was found between bacterial indicators *E. coli* and coliphages and five enteric virus types detected for the data collected during the Dusi Canoe Marathon race. This coincided with literature where a lack of consistent correlation between bacterial indicators and enteric viral pathogens was found (Love *et al.*, 2014; Girones and Bofill-Mas, 2013; Verani *et al.*, 2019). This coincides with literature where although bacteriological markers are usually present during viral outbreaks, faecal coliforms in recreational water sites are not always a sign of viral contamination (Li *et al.*, 2023). However, a statistically significant difference with moderate positive correlation ($r=0.578$, Table 8) was determined between *E. coli* and NoVGI ($p<0.00$, Figure 24) with a negative correlation found between *E. coli* and adenovirus during water quality monitoring for the Dusi Canoe Marathon. The highest *E. coli* value was found in lower the Kwapata region whereas the lowest was found in Msinsi Res at the end of day 2 of the marathon.

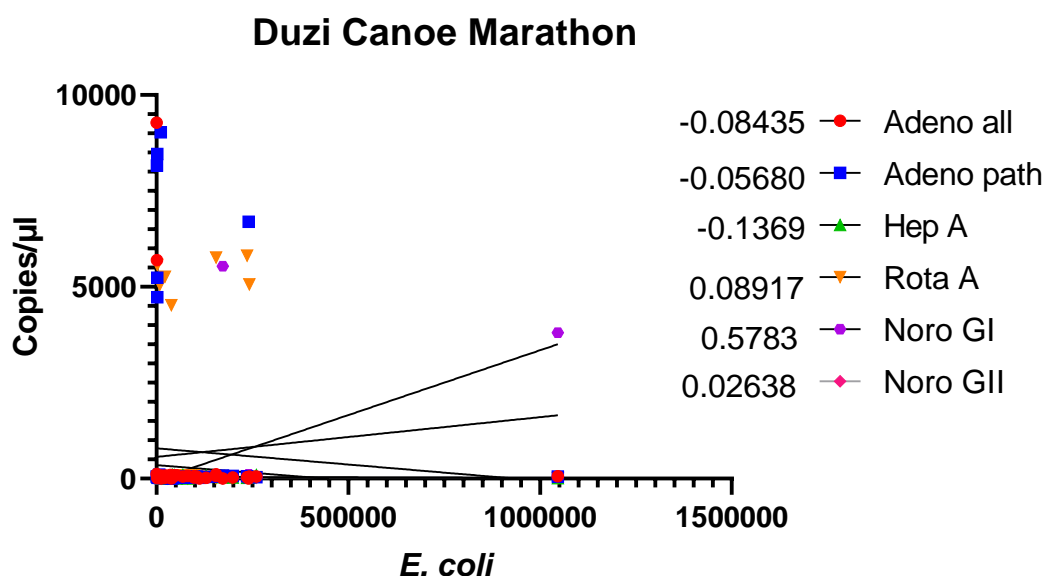


Figure 24. Significant associations between FIB and enteric viruses for the Dusi Canoe Marathon, 2021

Table 8. Correlation matrix between *E. coli* and enteric viruses detected during the Dusi Canoe Marathon, 2021

| | <i>E. coli</i> | <i>Adeno all</i> | <i>Adeno path</i> | <i>Hep A</i> | <i>Rota A</i> | <i>Noro GI</i> | <i>Noro GII</i> |
|-------------------|----------------|------------------|-------------------|--------------|---------------|----------------|-----------------|
| <i>E. coli</i> | 1 | | | | | | |
| <i>Adeno all</i> | -0,08435 | 1 | | | | | |
| <i>Adeno path</i> | -0,0568 | -0,057328917 | 1 | | | | |
| <i>Hep A</i> | -0,13685 | 0,173485477 | -0,055211391 | 1 | | | |
| <i>Rota A</i> | 0,089174 | -0,070023686 | 0,096591447 | -0,08797 | 1 | | |
| <i>Noro GI</i> | 0,578255 | -0,037110465 | -0,059243512 | -0,12042 | -0,06914 | 1 | |
| <i>Noro GII</i> | 0,026376 | 0,140810148 | -0,10523444 | -0,06893 | 0,071687 | -0,13197 | 1 |

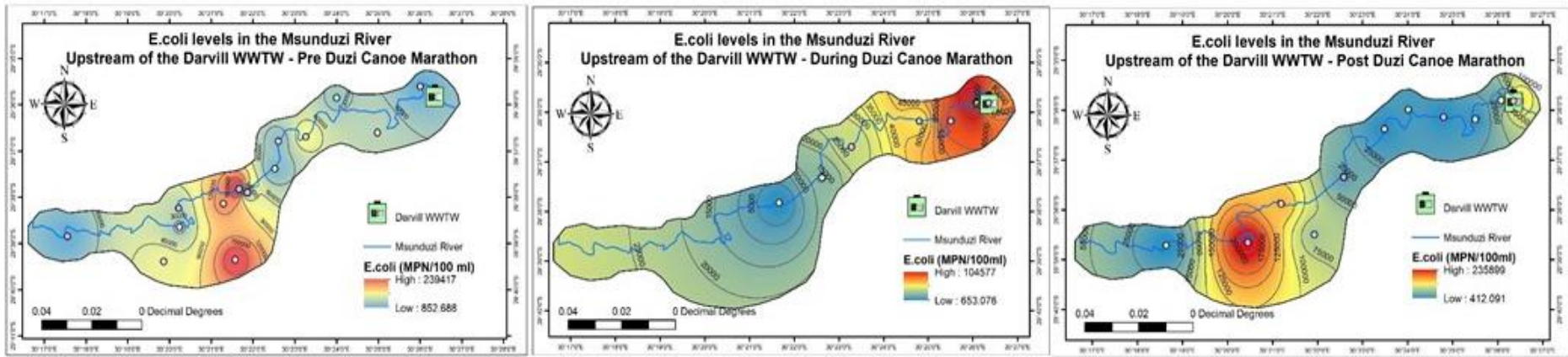


Figure 25. Distribution of *E. coli* contamination levels before, during and after the Dusi Canoe Marathon, 2021

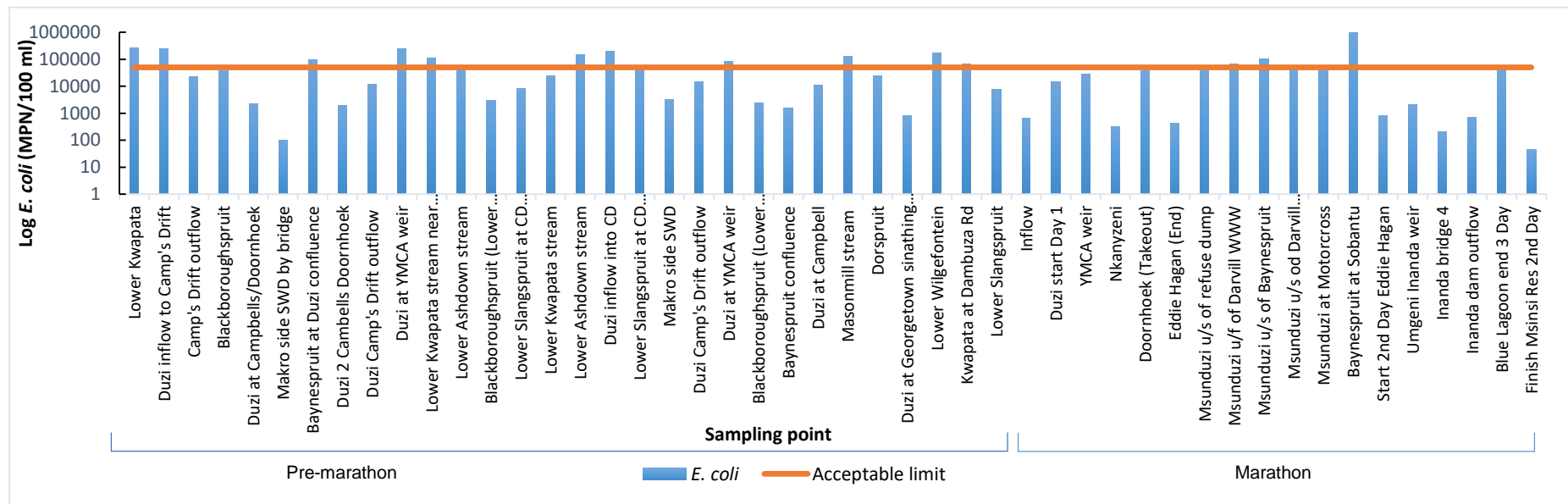


Figure 26. *E. coli* contamination levels before and during the marathon with concentrations exceeding >50 000 MPN/100 ml

The viral occurrence was not statistically significant in samples where bacterial load exceeded recreational limits of >50000 MPN/100 ml for *E. coli*. Recreational water does not undergo water treatment and at certain bacterial levels is considered acceptable for recreational activities. The presence of waterborne enteric viruses even at lower levels shows the potential public health burden. However, a study conducted by Masachessi *et al.*, (2021) proposed a pathway suggesting that if environmental waters exceed bacterial guideline limits, then the water can be classified as microbiologically hazardous and viral testing should not be conducted. They further went on to suggest that if the water body is within these limits, viral testing should be done, where human adenovirus testing will be used for MST as a human faecal indicator. In the current study, all enteric virus types tested were present in the samples that exceeded bacterial guideline limits of >50 000 MPN/100 ml as seen in Figures 25 and 26. No significant difference was found between adenovirus, a proposed viral indicator, and coliphages and between enteric viruses and *E. coli* as seen in Table 8. This was supported by research, which found that the prevalence of coliphages and human enteric viruses did not always coincide (Singh *et al.*, 2022). Therefore, coliphages being used as an indicator of enteric virus presence in water alone is not sufficient. Somatic coliphages are non-enveloped viruses making them structurally similar to enteric viruses. They are known to be present in high concentrations in wastewater and their removal during wastewater treatment processes has been reported as similar to that of enteric viruses (Sidhu *et al.*, 2018).

Using faecal coliforms as a means to predict the presence of rotavirus in the environment was not established as there was no correlation found between the two pathogens (Osuolale and Okoh, 2017). Bacteriological criteria have proven to be inadequate when evaluating the level of faecal pollution in water. The lack of these pathogens does not mean that enteric viruses—which are more resilient to sewage treatment processes than bacteria—are not present (Prez *et al.*, 2015). Between Figures 21 to 23 for enteric viruses and Figure 25 for *E. coli*, it can be seen that the highest concentrations, for both bacteria and enteric viruses, were obtained during the canoe marathon and closest to Darvill WWW. However, the highest average

concentration for AdV 40 and 41 and RVA was found before the marathon contradicting that anthropogenic activities have an impact on virus prevalence and survival as seen in Figures 21 and 22. This could be as a result of other anthropogenic activities other than the marathon such as a pollution discharge. Many people gathering for such an event require ablution facilities thus straining the wastewater treatment system therefore increasing the discharge. Alternatively, sediments are upset thus enabling the release of these viruses. This could also be a possible reason as sampling is done using grab samples and rarely is there sediment sampling.

4.3.4 Potential sources of enteric viruses in the Msunduzi River

A study confirmed that a combined sewage overflow contributed to the high concentrations of viruses in receiving waters (Fong *et al.*, 2010; Alejandro, 2016). This was more frequent during wet weather as compared to dry weather periods. Infectious enteric viruses present in final effluents and insufficiently treated wastewater have been demonstrated as sources of these viruses in the environment (Simmons and Xagorarakis, 2011; Pusch *et al.*, 2005; Haramoto *et al.*, 2008; Okoh *et al.*, 2010). The area surrounding each sample site provides a clearer understanding as to the potential sources of enteric viruses entering the Msunduzi River.

Sites 1 and 2, as seen below in Figure 27, were chosen as these were generally the lowest contamination sites for *E. coli* based on historical trends. Water is released from the small impoundment known as Henley Dam for the canoe marathon for increased river flow.

When there are rain events, there is a potential for the faecal matter of human and animal sources to enter into the river. However, due to the distance between Site 1 and Site 2, there should be natural removal, die off and settling out of enteric viruses before Henley Dam. Henley Dam itself will act as a removal mechanism similar to Camp's Drift. No statistically significant difference was found between *E. coli* and enteric viruses in Site 1, however, there was a positive correlation found between *E. coli* and NoVGII. *E. coli* did however have a strong positive correlation with rainfall ($r=0.896$) and a moderate positive correlation with turbidity

($r=0.518$). Henley Dam was considered a key control system in this study because the distance between those two sites is not very large and the dam is usually full. It is quite small if there is a large flow of water, hence the need for turbidity data. In this study, Site 2 showed elevated turbidity coinciding with rainfall events. This is possibly due to a plug of water rushing through from rain events in the catchment which brings contaminants with it, unlikely washing through the dam. In the majority of cases, any dam would act as a good control for *E. coli*. In a study done by (Goh *et al.*, 2019), turbidity was found to correlate with bacteria and coliphage concentrations but not with enteric viruses.

Downstream of Site 1 and Site 2 is the Edendale area, where there are various tributaries and there is a very similar area to the area above Henley with a growing population in the form of a town. Mabane is similar to the Edendale area set-up and is part of Vulindlela as well. There are some fully sewerred systems through the Georgetown area and better water supply coming into these areas where some of them have full flush toilets. However, they may not have any direct connection to the sewer network. Therefore, they are looking at septic tanks and conservancy tanks which require vacuum tankers to come and remove the waste. There are some areas of this, like Georgetown in particular, that are directly connected to the main sewer line and that sewer line is considered a major problem contributing as a potential source of enteric viruses.

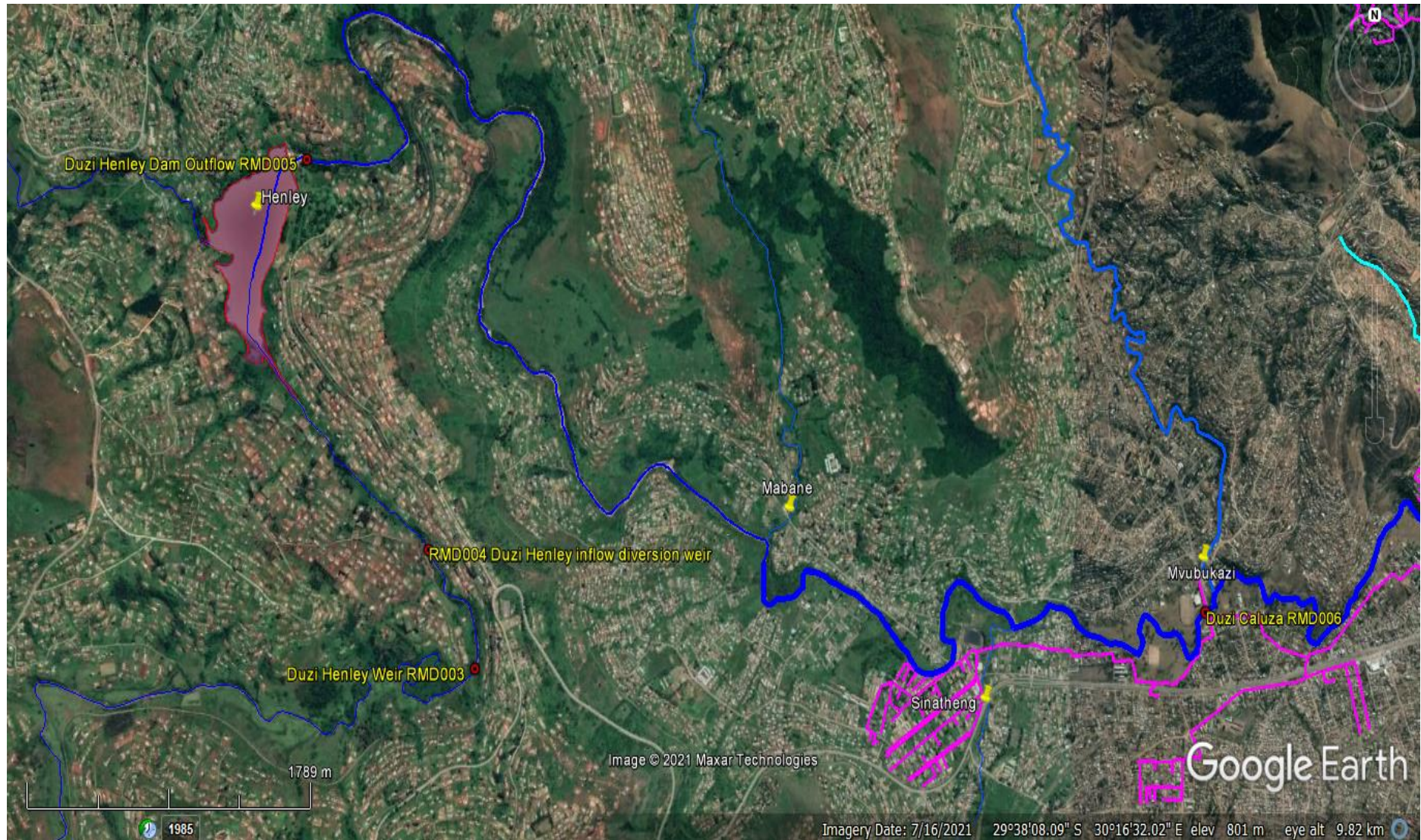


Figure 27. Sampling sites 1, 2 and 3 in the Msunduzi River



Figure 28. Surrounding area and sewer network of Duzi at Caluza sampling site 3

At Sampling site 3, the Caluza site, the average *E. coli* contamination levels were found to be 190186,90 MPN/100 ml which was excessively high. This was due to a sewer line running there as seen in Figures 27 and 28. This coincides with the overall highest concentration of AdV found at this site which was also affected by a rainfall event. The main sewer network also collects from the Mvubukazi side as well where there is a sewer line running with gravity the same way as the river. There is a main connection point there as well as seen in Figure 28. The sewage coming out of the manholes drains into the river just before the sample site. If the polluted water has aged and the source of contamination is far from the sample site, the virus concentration determined by molecular techniques may significantly overstate the infectious concentration (Li *et al.*, 2023).

The Ashdown stream has multiple problems where the sewer network runs closer to the stream where sewer contamination is evident. There are also sewer crossings and a manhole in the stream channel which will cause a problem all of which flow into the Duzi above Site 4. This site, however, also collects problems from the KwaPata area into the KwaPata stream where Edendale Hospital is in the same area as seen in Figure 29. Manholes can be problematic in the KwaPata system which is rather poor and if there is also hospital waste present in it then the likelihood of having sick people there is somewhat high. The surrounding community will be exposed to waste excretions from sick patients, which will cause infections via direct contact or aerosols. This can then contribute to the situation at sample site 4. There are a fair amount of domestic animals in the area and there is an informal settlement, namely Peace Valley. The likelihood of surface runoff during rainfall events into the river is high. Also to a certain extent further back from these areas where there are formal areas, but pit latrines and not sewers, rain runoff and pit latrines that are not adequately cleared there will be active waterborne pathogen transport from those. These areas are much more formalised, but they are not necessarily connected to sewer systems. The open areas are heavily grazed by cattle and goats which are defecating directly onto the land surface which is then associated with rain transport and higher turbidity when going into a collector system like the KwaPata system.

Viral transport is also a time-related issue. For example, if there is a huge sewer problem going into the Duzi, it may be detected in Site 3 but if this occurs in winter, it may take 2 days for the water to be transported to Site 4. Even if there are no other inputs from the intervening intermediate area, what was in Site 3 is likely to be acted upon by the river effects, UV kills for *E. coli* and inactivation of viruses before it gets to Site 4. Another factor that can impact viral transport, concentration and detection is the surrounding industries around the sample sites such as Edendale Hospital, Edendale Mall or the fuel and oil industries in the area. A viral surveillance study conducted by Hellmér *et al.*, (2014) indicated that norovirus and hepatitis A detected in sewage could be related to hospitalised patients infected with the virus in the catchment area of the sewage system. This confirmed the need to shift the surveillance scope of waterborne viruses from the hospital to the environment.

The main industries of Pietermaritzburg are just below Site 4 and there are other industries present in the Slangspruit area, which will be likely to affect Site 5. Camp's Drift acts in the same manner as Henley Dam as seen in Figure 30. The *E. coli* inputs from Site 4 and Slangspruit were high, however, the top end of Camp's Drift serves as a pre-treatment facility because it acts as a wetland. There is a lot of sediment input from upstream, but it acts as a sewage treatment plant where residence times are high. Due to high residence time, enteric pathogens will be exposed to UV inactivation, dilution and increased settling out of sediments thereby increasing the removal rate of viruses in Camp's Drift.

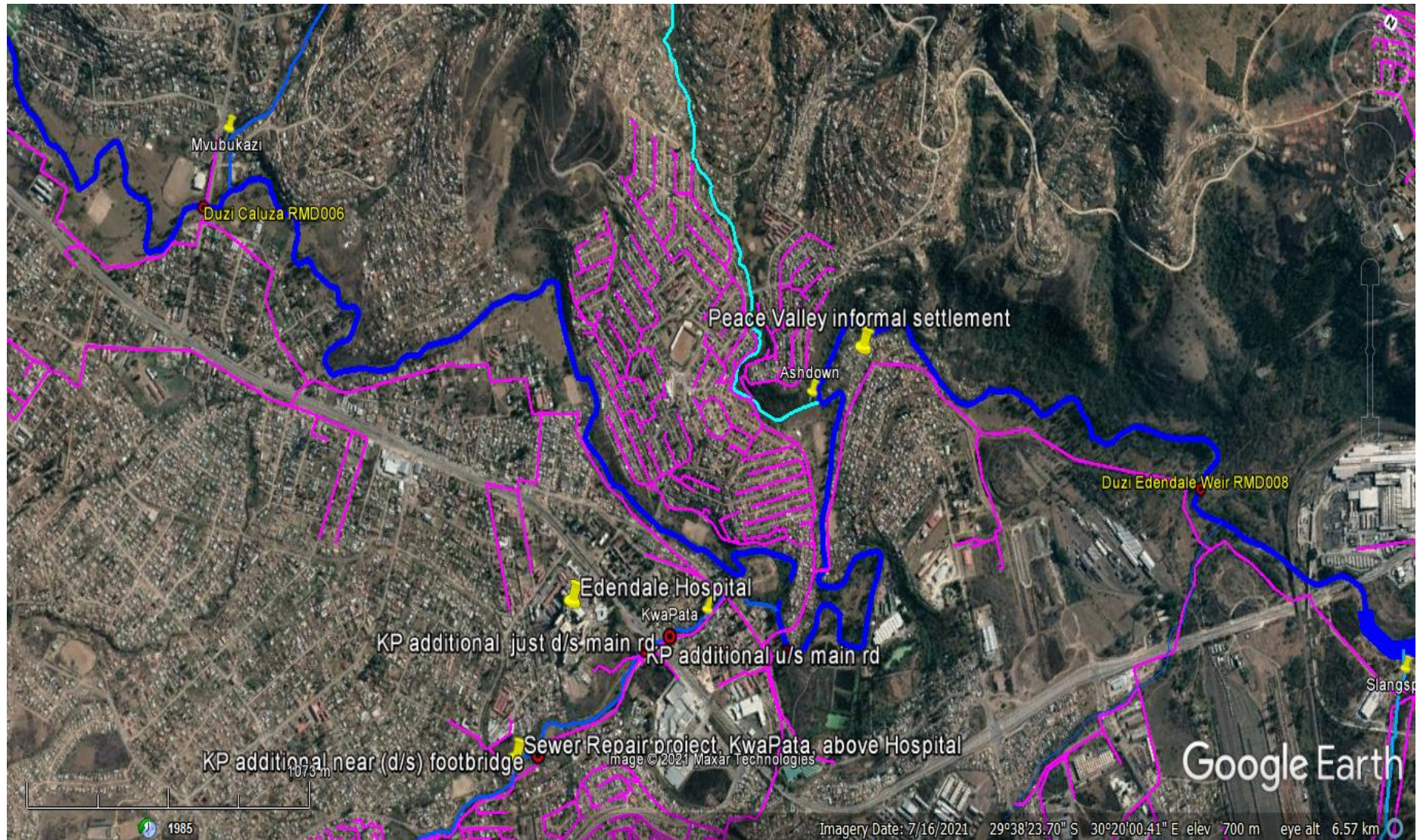


Figure 29. Surrounding area and sewer network of Duzi at Edendale weir sampling site 4

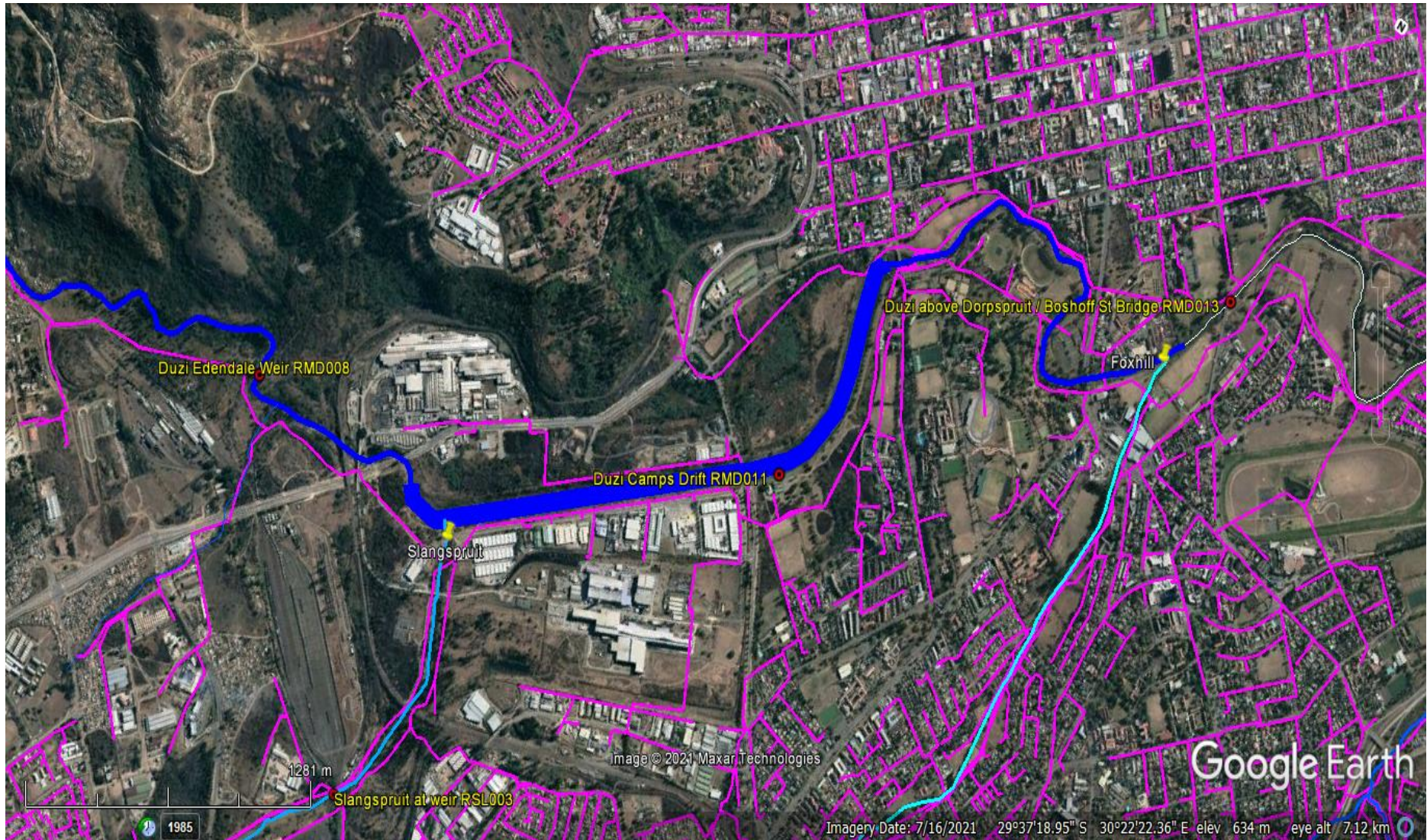


Figure 30. Area surrounding Site 4 and Site 5 depicting canalised Camp's Drift

This then leads to Site 5 as seen in Figures 30 and 31. Camps Drift serves as an effective pathogen removal site however this may be undone by the potentially bad effects of Pietermaritzburg CBD inputs which contain high concentrations of pathogens entering the river. This part of Pietermaritzburg and the Foxhill spruit tributary area is formerly sewered. There is a footbridge and a large stormwater drain that comes in from below the old hospital which drains a large part of the CBD into the Duzi. There are also many sewers being connected that now run alongside the Duzi. The connections are running in parallel and are intended to run that way but if one is leaking or surcharging then this becomes a cause for concern. The other sewer line runs to the YMCA weir. The Duzi race samples show that these areas have issues within the sewer network as it is undersized, especially for the recent population growth. Unlike the CBD, there can be sewer problems here which are more frequent and sometimes small but cumulative and may not be addressed adequately and promptly by the municipality. The sewers in this area were meant to be replaced by newer, much larger ones and because this hasn't been done it has contributed to the contamination cases. Often Site 5 is of much better water quality because it is a formerly sewered area and it has Camp's Drift upstream. Virus concentrations were significantly lower for HAV in Site 5 as compared to Site 4.

There is a slight difference from Site 5 to Site 6, as depicted in Figure 31, due to the Dorpspruit and the Ash Road squatter settlement. There is high-intensity housing even though it is formally sewered there has been a significant increase in the population with a relatively limited sewered system which can cause problems. Electricity theft in this area in particular is affecting sanitation because it has made access to the sewer network almost impossible. Therefore, if there is a sewer problem here, the chances of it getting fixed are remote. The Dorpspruit area contributes to higher sewer flows.

Communicable diseases commonly associated with the gastrointestinal tract have been linked with recreational swimming, contaminated bathing sites and wading (Prez *et al.*, 2015). Although the bacterial water quality deteriorated along the river course, the concentrations of

enteric viruses did not indicate any significant difference among the sampling sites. This may be due to the sampling method where grab samples were taken. Sediment sampling may have revealed a different result as virus particles are known to attach to sediment particles allowing them to survive for longer periods of time in water.

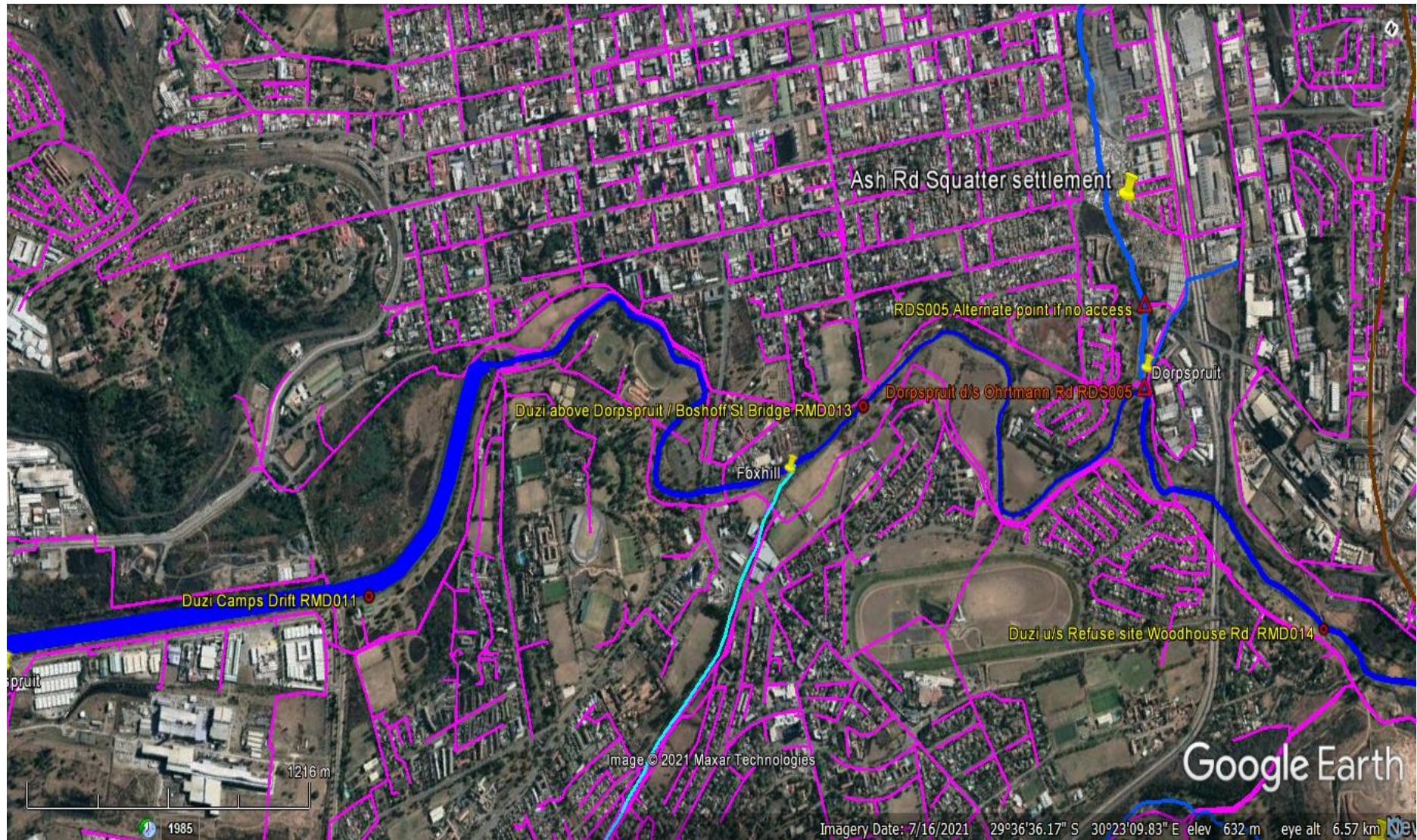


Figure 31. Surrounding area and sewer network of sampling sites 5 and 6

4.3.5 Potential viral indicators

A study (Sidhu *et al.*, 2018) suggested that human adenovirus be used as an indicator of the efficiency of enteric virus removal due to its high prevalence in both influent and effluent and its low removal rate. The groups of viruses proposed as water quality indicators have great diversity, hence a single virus that meets the requirements of a viral indicator has not yet been found. Local or regional viral indicators need to be investigated as the presence of some enteric viruses in the environment depends on local epidemiology (Masachessi *et al.*, 2021). Another study confirmed the use of AdV as a viral indicator due to it being present in surface waters all year round independent of seasonal change (Opere *et al.*, 2020). Specifically, compared to other microbes that are frequently utilised as indicators for water quality assessment, human enteric viruses are highly persistent in aquatic environments. The pattern of human infections has been demonstrated to be closely correlated with the presence and diversity of these viruses (Hess *et al.*, 2021).

All samples analysed in this study contained Adenovirus with 100% of samples testing positive for pathogenic adenovirus types 40 and 41. Adenoviruses were present in all samples that contained *E. coli*, including the lowest value of 47 MPN/100 ml. This confirmed that adenovirus is a suitable indicator for the presence of faecal contamination in comparison with bacterial indicators such as coliforms and coliphages. The efficiency of using bacteria as indicators of viruses is questionable due to their differences in the life cycle, structure, survival and persistence in environmental waters (Pascual-Benito *et al.*, 2020). Adenovirus can be used as indicator for the presence of other enteric viruses, as it was indeed detected in every water sample that RVA, HAV, AdV 40/41, NoVG I and NoVG II were detected in. The concentrations of adenovirus detected was much higher than the other enteric viruses tested for, in most cases. An alternative indicator or group of indicators will need to be investigated to determine the presence of enteric viruses in a riverine environment. Based on the results, it can be said that the adenovirus assay for all serotypes can be used to determine the presence of AdV 40

and 41 strains as each sample that tested positive for adenovirus tested positive for pathogenic adenovirus as well.

Rotavirus has been recommended as a viral indicator, however, the high presence of faecal coliforms and low concentrations of rotaviruses indicate that it would be a poor indicator of faecal contamination events as demonstrated by (Osuolale and Okoh, 2017). In this study, these results were confirmed where poor to negligible positive and negative correlations were observed between RVA and *E. coli*. Rotavirus A showed no significant associations with any of the other enteric viruses tested for. There were moderate ($r=0.540$) and strong positive ($r=0.834$) correlations with NoVGII in sites 5 and 6 respectively. Moderate ($r=-0.618$) and strong negative ($r=-0.853$) correlations with NoVGI were observed in sites 2 and 3 respectively. Pepper mild mottle virus (PMMoV) has also been recommended as a viral indicator as it has shown greater stability and persistence during wetland treatment as opposed to human enteric viruses (Rachmadi *et al.*, 2016). Future research should focus on its applicability as a viral indicator in water.

Somatic coliphages have been routinely used in recreational water quality assessments. This is due to their persistence in environmental waters being a close resemblance to viral pathogens owing to their similar structural and morphological characteristics. It was found that somatic coliphages decay at a similar rate to infectious adenovirus in environmental waters (McMinn *et al.*, 2020). However, the results in this study did not corroborate; very strong to moderate negative correlations were observed across sites 1 to 5 between NoVGI and somatic coliphages. A moderate positive correlation was found with RVA in sites 3 ($r=0.523$) and 4 ($r=0.602$). Very strong positive correlations ($r=1$) were observed between NoVGII and somatic coliphages in sites 1 and 2. In this study, no significant associations were found between somatic coliphages and the enteric viruses tested for. Therefore, they were not considered to be a useful indicator to predict the presence of enteric viruses in a riverine environment.

4.3.6 Potential risk assessment

The Water Research Commission (WRC) has proposed risk-based guidelines for recreational water use that have improved on the South African Recreational Water Quality Guidelines of 1996 (Genthe, Claassen and Steyn 2020). The *E. coli* levels proposed in the new guidelines are much lower than those proposed by DUCT for the annual Dusi Canoe Marathon as seen in Table 11.

According to the US EPA, the illness rates associated with Recreational Water Quality Criteria are between 32 to 36 gastro-intestinal illnesses per 1,000 primary contact users or approximately 3.4%. The measuring system used by the WHO to evaluate public health risk and disease burden assessment associated with environmental water exposure is the disability-adjusted life year (DALY). The DALY takes into account the number of years lived with a disability (YLD) and the number of years of life lost (YLL) due to the hazard. Calculation of the DALY contribution per infection can be made using the following formula:

$$\text{DALY} = \text{YLL} + \text{YLD}$$

The DALY can be utilised in defining the tolerable burden of disease and the related reference level of risk as seen in Table 9. It is important to note that norovirus was used in the new guidelines as the reference viral indicator.

Table 9. Estimates of Hypothetical Disease Burden by different waterborne pathogens adapted from WHO, 2016 (Genthe *et al.*, 2020)




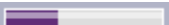
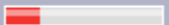


| Pathogen | Disease burden per 1000 cases | | |
|---|-------------------------------|------|------|
| | YLD | YLL | DALY |
| Cryptosporidium | 1.34 | 0.13 | 1.47 |
| Campylobacter | 3.2 | 1.4 | 4.6 |
| Shiga-toxin producing <i>E. coli</i> | 13.8 | 40.9 | 54.7 |
| Rotavirus | | | |
| High income countries | 2 | 12 | 14 |
| Low income countries | 2.2 | 480 | 482 |
| Hepatitis A | | | |
| High income countries | 5 | 250 | 255 |
| Low income countries | 3 | 74 | 77 |

The probable risk associated with canoeists can be found in Table 11 where *E. coli* values greater than 50000 MPN/100 ml were found in 29.27% of the samples taken. The actual risk associated with ingestion of contaminated water by canoeists can be modelled using QMRA which is a sensitive tool that can estimate the probability of infection which was unable to be measured through epidemiological studies but can be used to complement it. Risk assessments can be used to predict infection or illness rates based on the measured or predicted density of a specific pathogen or in this case enteric virus ingestion rates with associated activities. The new guidelines assumed an ingestion rate of 4 ml per hour for canoeists. The risk of Nov infection was modelled by the levels of *E. coli* present in the water which was used to predict the norovirus concentration as seen in Table 10. A study, using an integrated approach combining a life cycle assessment and QMRA, revealed the annual probability of illness per individual was the highest for norovirus and adenovirus (Bhatt *et al.*, 2023).

Table 10. Reference pathogen formulae and data to calculate DALYs adapted from WRC Guidelines, 2020

| Reference pathogen | Campylobacter | Norovirus | Cryptosporidium |
|--|---|--|----------------------------------|
| Dose-resoponse parameters | $\alpha = 0.145$ $\beta = 7.58$ | $\alpha = 0.0044$ $\beta = 0.002$ | r=0.2 |
| | Approx beta Poisson | Hypergeometric | Exponential |
| Low-dose extrapolation formula | $P_{inf} = \frac{\alpha}{\beta} \times \text{dose}$ | $P_{inf} = \frac{\alpha}{(\alpha+\beta)} \times \text{dose}$ | $P_{inf} = r \times \text{dose}$ |
| Probability of infection from a single organism | 0.019 | 0.69 | 0.7 |
| Likelihood of becoming ill if infected | 0.3 | 0.7 | 0.7 |

Table 11. Assessment of risk posed to canoeists based on *E. coli* present in the Msunduzi River adapted from DUCT

| <i>E. coli</i> MPN/100 ml | Water quality “assessment” | Assessment of probable risk to canoeists |
|------------------------------|---|---|
| 0 – 130 |  Excellent  Good | Excellent – Considered acceptable for full contact recreation – excellent/likely natural catchment. |
| 130 – 1000 | | Good – within the range expected for quite good catchment conditions, but significant / increasing risk of illness if used for full contact recreation (e.g. swimming). |
| 1001 – 4000 |  Fair | Fair resource conditions, natural sources (i.e. not sewage-related) are still possibly the <i>E. coli</i> source, but the increasing likelihood of human faecal / sewage contamination at the upper end of this range. Unacceptable for swimming. |
| 4 001 – 10 000 |  Poor | Degraded – Significant faecal input or some sewage contamination is indicated. Other water quality parameters are likely to be poor. |
| 10 001 – 25 000 |  Very Poor | Unacceptably Poor, sewage contamination is definitely present. |
| 25 001 – 50 000 |  Dangerous | Bad, with significant sewage contamination and negative environmental consequences. |
| >50 000 |  Very Dangerous | Severely contaminated with significant sewage contamination and negative environmental consequences. |
| | | Extremely low risk. |
| | | Very low risk. |
| | | Low, but increasing risk. |
| | | Moderate risk (approx. 1 in 10 may get sick). |
| | | Moderately high canoeing risk (approx. 1 in 5 may get sick) – some likelihood of illness esp. in white water. |
| | | High risk (approx. 1 in 3 may get sick) – consider not canoeing or paddling on flat water only, with no risk of falling out. |
| | | Very High canoeing risk – (approx. 1 in 2 paddlers may get sick). Avoid canoeing! |

4.4 Conclusion

This study examines the prevalence and distribution of pathogenic bacteria, enteric viruses, and microbial indicators in surface waters taken from six sampling locations with different land uses during a seven-month period in the Msunduzi River in Pietermaritzburg. Molecular detection techniques were used to detect a variety of pathogenic enteric viruses quickly and simultaneously. There were either negligible or no relationships between indicators and viral infections, despite the substantial associations between FIB and coliphages. The present study identified adenovirus as a universal indicator that was associated with all the pathogens tested. Furthermore, it was determined that environmental parameters including temperature, land use, rainfall, geographic location, etc., could have a significant impact on the correlations between indicators and pathogens. As a result, rather of using only one indicator, future pathogen prediction may need a suite of markers. Further research is needed from various geographical locations around the world to assess the potential for using these indicators as pollution indicators, as many factors could affect their survival and persistence in surface water samples.

Using models to estimate the risk associated with recreational water activities is a more feasible approach as compared to enteric virus detection methods. However, more accurate models can be done when using actual viral data versus bacterial indicators to predict the presence of enteric viral pathogens. Since it is impractical to conduct QMRA on every waterborne enteric pathogen, an index pathogen or group of index pathogens ought to be selected.

CHAPTER FIVE – CONCLUSIONS AND RECOMMENDATIONS

- Digital PCR was found to be a suitable method for the detection of enteric viruses in a riverine environment.
- Statistically significant associations were found between enteric viruses and pH, rainfall, TOC, Al, NH₃, total phosphorous, turbidity and suspended solids at more than one sample site revealing the possible impact physiochemical parameters have on the survival of enteric viruses.
- Sole reliance cannot be placed on bacterial indicators as water quality indicators as there were either negligible or no relationships between indicators and viral infections.
- Adenovirus is a proposed universal indicator as it was detected with all the enteric viruses tested for in this study.
- Anthropogenic activities and seasonal changes had an impact on the concentration of enteric viruses detected in the Msunduzi River.
- Further research should be undertaken to determine a suite of markers for pathogen predication rather than reliance being placed on one indicator.
- Viral data collected should be used to develop a model for QMRA to predict the presence of enteric viral pathogens using index pathogens instead of conducting an assessment per virus.
- Using statistics, with the generation of more data, physiochemical predictors may possibly be found to predict the presence of enteric viruses in a riverine environment.

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APPENDICES

6.1 Appendix A Reagent preparation for virus filtration and concentration

Phosphate Buffer Saline (PBS)

PBS 1X tablets were dissolved in molecular grade water according to manufacturer's instructions. Careful attention was paid to homogenisation. Aliquots of 20 ml were made thereafter and stored at room temperature for 2 weeks after the first use of the aliquots.

Polyethylene Glycol (PEG) 50%

A volume of 500 g of PEG 6000 was prepared in 450 ml of molecular grade water and heated to dissolve under agitation at a maximum of 50°C. The solution was then made up to 1 L. Aliquots of 500 ml were made and sterilised at 121°C for 15 minutes and thereafter stored at room temperature.

Chloroform/Butanol solution

This solution was made in a 1:1 ratio where 50% chloroform (CHCl_3) and 50% n-Butanol ($\text{C}_4\text{H}_{10}\text{O}$) were added together. This was performed under a fume hood due to the aeration reaction caused. The solution was stored at room temperature for up to 12 months.

Glycine Buffer

A volume of 9 g NaCl and 3.75 g Glycine was prepared in 800 ml of molecular grade water. The pH was adjusted to 9.5 and then completed to a final volume of 1 L. Aliquots of 500 ml were made and sterilised at 121°C for 15 minutes. The buffer was then stored at room temperature.

6.2 Appendix B Sampling

Table 12. Sampling sites and co-ordinates in the Msunduzi River, Pietermaritzburg

| Site | Sample Point | Sample Description | Degrees South | Degrees East |
|------|--------------|---------------------------------------|---------------|--------------|
| 1 | RMD 003 | Duzi Henley weir | 29 38 49.60 | 30 15 34.78 |
| 2 | RMD 005 | Duzi Henley outflow | 29 37 23.9 | 30 14 53.5 |
| 3 | RMD 006 | uMsunduzi at Caluza bridge | 29 38 42.6 | 30 18 38.2 |
| 4 | RMD 008 | uMsunduzi at Edendale weir | 29 37 52.3 | 30 21 11.3 |
| 5 | RMD 013 | uMsunduzi above Dorpspruit confluence | 29 36 22.6 | 30 23 29.2 |
| 6 | RMD 014 | uMsunduzi above refuse dump | 29 36 08.1 | 30 4 47.8 |

General U UW procedure for impoundment sampling

- Do not go onto an impoundment that is spilling unless you can launch the boat and stay on the opposite side of the buoy line to the wall.
- Radios and or cell phones must be taken onto the impoundment.
- Do not drag rope/cable over the railings
- DO METERS MUST BE CHARGED AND CHECKED BEFORE LEAVING.
- Check that the appropriate length of DO cable for the particular impoundment is taken.
- Where possible, take 2 DO meters on every run. Please take every care of the meters.
- DO NOT tamper with the membrane or meter to get it to work, rather make several attempts to calibrate and if not successful contact the SSMP Coordinator, or SSMP Scientific Assistant
- Always take the pH readings and then the bacteriological samples first, whether directly or from a bucket sample, to avoid possible contamination.

- The surface samples must not be skimmed but taken approximately 5cm below the surface.
- Look out for contamination (e.g. oil from the motor, sampling where any remaining sample was dumped).
- Rinse buckets with actual sample from the depth sampler (not only with surface water).
- **The integrated sample must not be taken when the depth at the sample point is less than 4.5 m.**
- **The floor sample must not be taken when the depth at the sample point is less than 3m.**
- DO and temperature profiles must always be done because of possible water quality problems.
- Observe all Safety Regulations.
- Make sure that there are life jackets; boot-chest waders, gumboots or safety shoes are worn.
- Always report to the Superintendent before going onto the impoundment.
- If for some reason one cannot launch the boat, samples must then be taken from the impoundment wall and a comment entered on the round sheet and in the dam book.

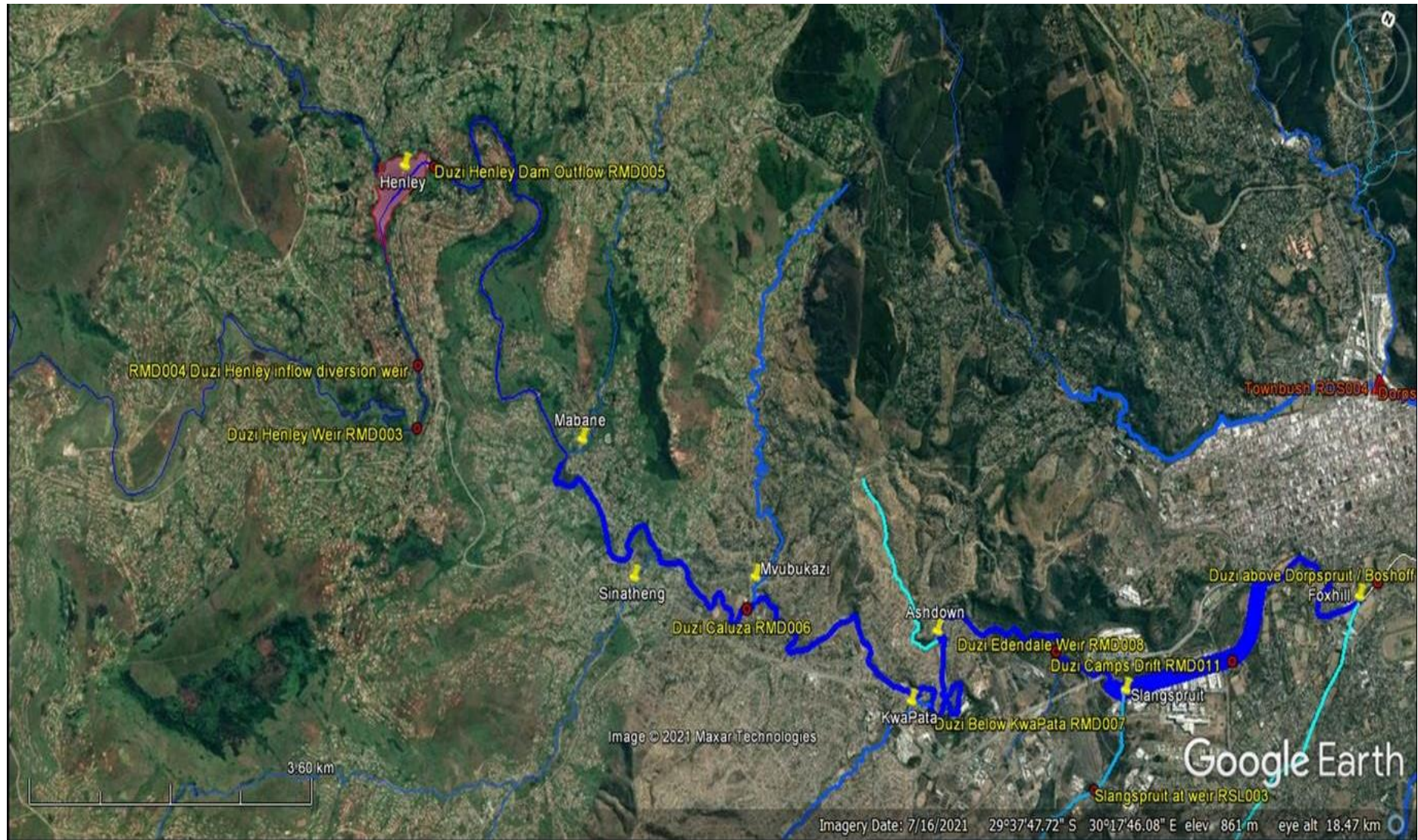


Figure 32. Sampling sites upstream of Msunduzi River, Pietermaritzburg

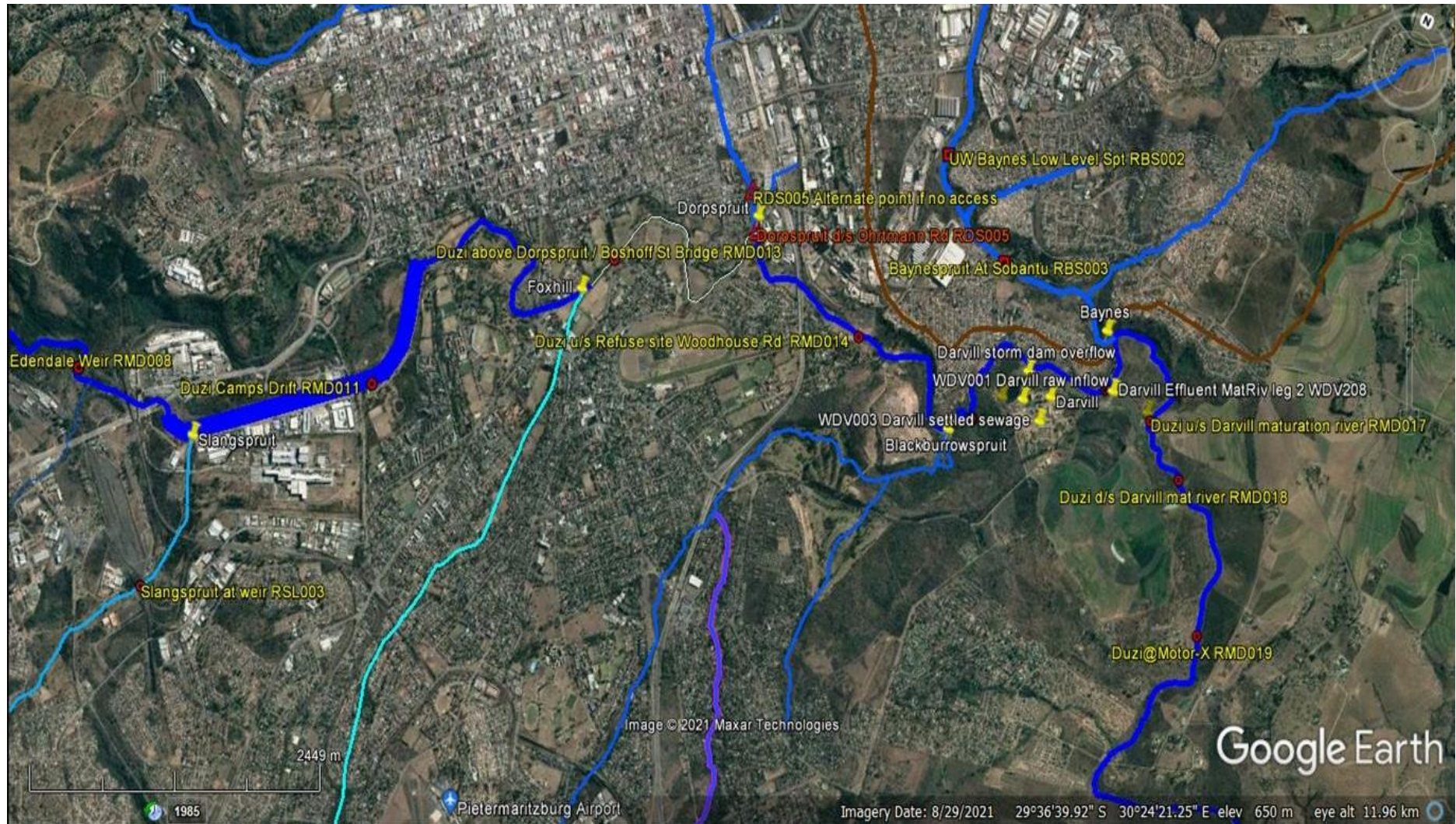
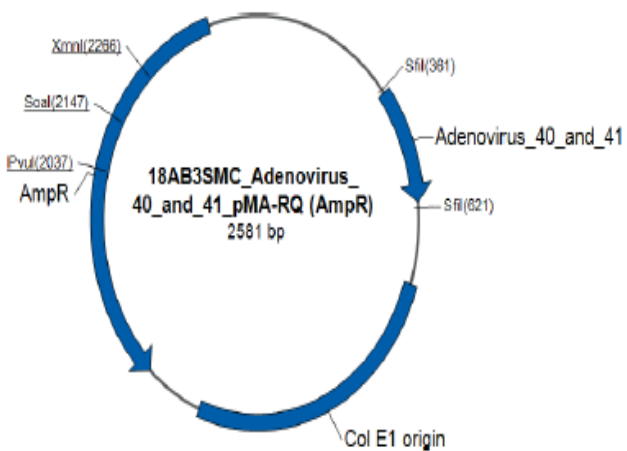
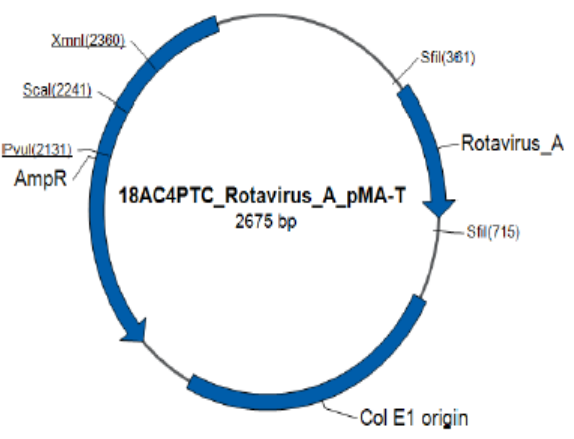
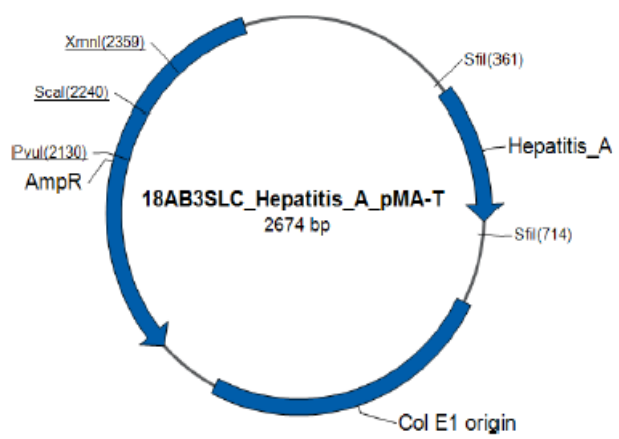
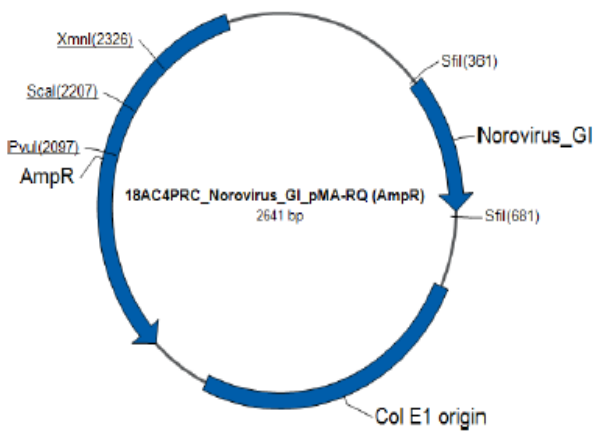
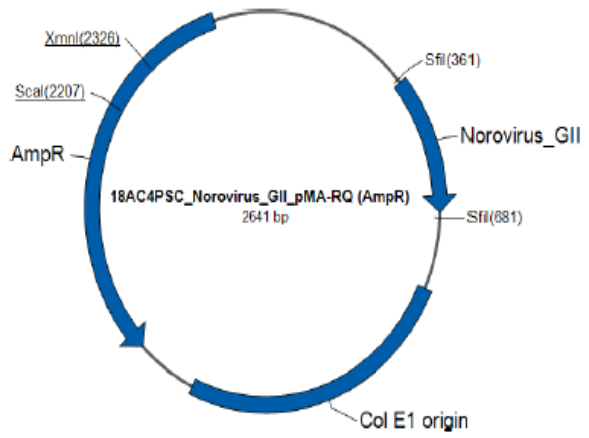


Figure 33. Sampling sites downstream in the Msunduzi River, Pietermaritzburg

6.3 Appendix C Plasmids used for quality control



6.4 Appendix D MagMAX

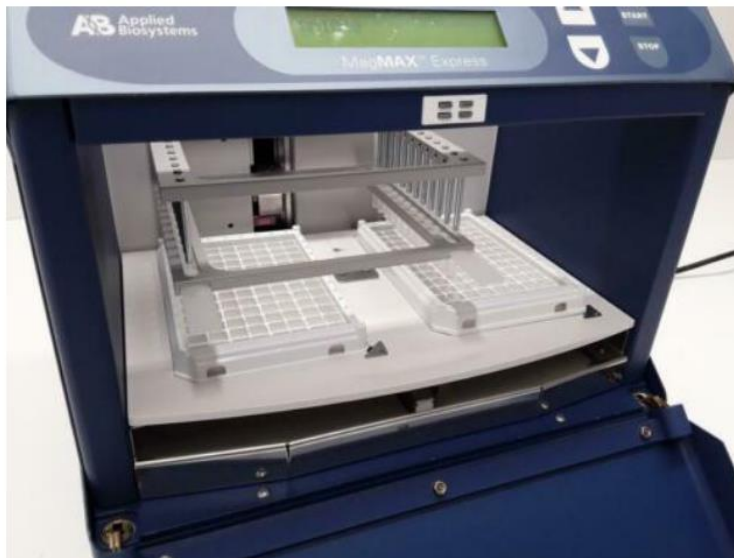


Figure 34. MagMAX xpress instrument (Life Technologies)

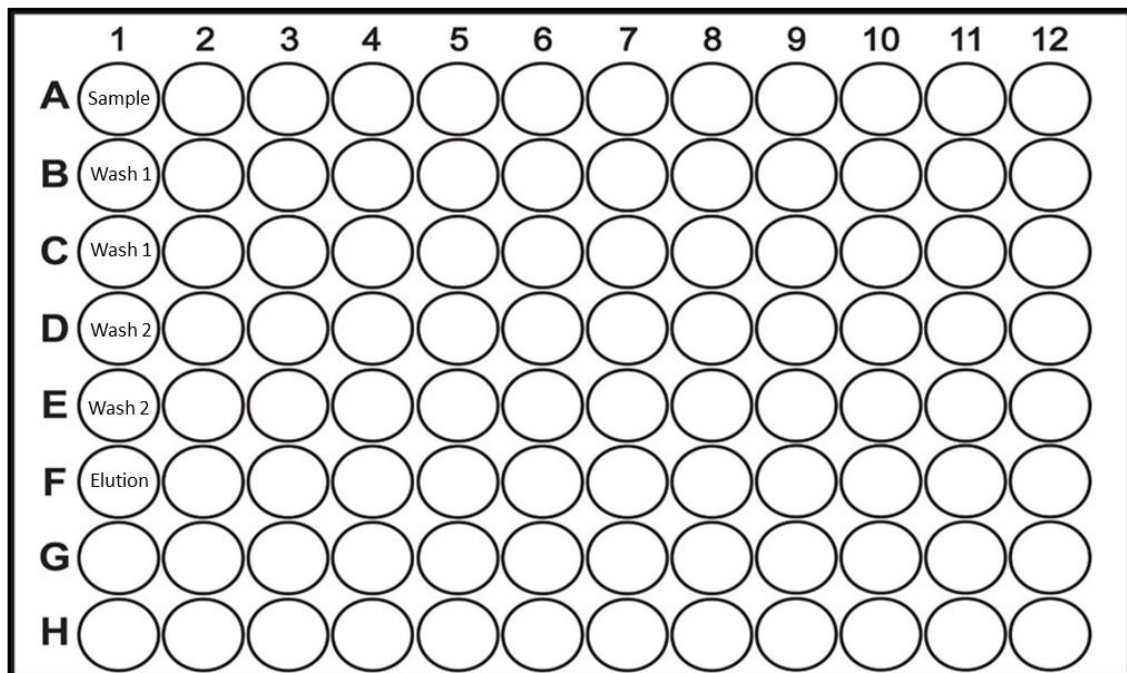


Figure 35. 96 well processing plate layout for nucleic acid extraction

6.5 Appendix E Physiochemical characterisation methods

pH and temperature

It is important to stipulate the temperature at which the pH was taken as pH values are temperature dependent. A Meter Lab PHM220 pH meter equipped with a pH electrode and a temperature compensation probe was used. The pH meter was calibrated before use with buffers 4.00, 7.00 and 10.00 and quality controls were conducted before the pH of the samples was measured. All pH and temperature values for the samples were measured on-site, immediately after sampling, as the pH is likely to change with the potential dissolution or absorption of carbon dioxide to or from the atmosphere. At least 50 mL of the sample was poured into a beaker and used for analysis. This method was carried out as per the Standard Method 4500, 23rd edition.

Turbidity

Turbidity is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. Turbidity in water is caused by suspended matter, such as clay, silt, finely divided organic and inorganic matter, soluble coloured organic compounds and microscopic organisms and thus may be a useful parameter in virus survival assessment. This method is based on a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension under the same conditions. The higher the intensity of scattered light, the higher the turbidity of the sample. Formazin polymer was used as the primary standard reference suspension. This method was carried out according to Standard Method 2130, 23rd edition.

The instrument used was a 2100AN turbidimeter, a nephelometer that has a light source for illuminating the sample and detectors placed to measure the 90° light scatter to the path of the incident light. The second instrument was the TU5200 turbidimeter which measures turbidity by directing a laser into a sample to scatter off suspended particles. The light that is

scattered at 90° from the incident beam is reflected through a conical mirror in a 360° ring around the sample before it is captured by the detector. The turbidity was measured in nephelometric turbidity units (NTU) based on formazin standards. The working ranges for the 2100AN are between 0.200 NTU and 4000 NTU and the TU5200 between 0.200 NTU and 600 NTU.

Biochemical Oxygen Demand (BOD)

The BOD determination measures the oxygen required (by organisms already present in the sample) for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidise inorganic material, such as sulphides and ferrous iron. It may also measure the oxygen used to oxidise reduced forms of nitrogen unless an inhibitor prevents their oxidation. Samples for BOD analysis may degrade significantly during storage between collection and analysis, resulting in low BOD values. To minimize this, the sample was analysed within 6 hours of collection or kept at or below 10°C until analysis could be carried out. Analysis was started no later than 24 hours after the collection of samples. A 5-day BOD measurement was utilised.

The method required an air-tight bottle, 250 ml to 300 ml capacity glass bottles with ground-glass stoppers, to be filled with the sample. Parafilm was placed over the stopper to reduce the evaporation of the water during incubation. The sealed bottle was then incubated excluding light at room temperature for 5 days to prevent the possibility of photosynthetic production of Dissolved Oxygen (DO). The DO was measured before and after incubation. The BOD was then calculated from the difference between the initial and final DO readings. Samples, such as wastewaters, contain more oxygen-demanding materials than the amount of DO available in air-saturated water. Therefore, it was necessary to dilute the samples to bring the oxygen demand and supply into appropriate balance. Bacterial growth requires nutrients such as nitrogen, phosphorus and trace metals. Hence, these nutrients were added to the dilution water, which was also buffered to ensure a pH range suitable for bacterial growth. The method was carried out according to Standard Method 5210, 20th edition.

Calculation of results

When the dilution water is not seeded: $\text{BOD mg/L} = (D1 - D2) / P$

When dilution water is seeded: $\text{BOD mg/L} = ((D1 - D2) - (B1 - B2)f) / P$

Where: D1 = Initial DO of sample

D2 = Final DO of sample (i.e. after 5 days incubation)

P = Decimal volumetric fraction of sample used (i.e. for 25% dilution, P is 0.25)

B1 = Initial DO of seed control

B2 = Final DO of seed control (i.e. after 5 days incubation)

f = Ratio of seed in sample to seed in control = $(\% \text{ seed in D1}) / (\% \text{ seed in B1})$

When the sample is seeded: $\text{BOD mg/L} = f(D1 - D2) - (f - 1) / f (B1 - B2)$ where $f = V2 / V1$

Where: D1 = Initial DO of sample.

D2 = Final DO of sample (i.e. after 5 days incubation).

B1 = Initial DO of seeded dilution water.

B2 = Final DO of seeded dilution water.

V1 = Sample aliquot (before dilution).

V2 = Final volume of seeded sample.

Total Organic Carbon (TOC)

The organic carbon in water and wastewater is composed of a variety of organic compounds in various oxidation states (including those present in any living or viral components in the sampled water). While this is used as a non-specific indicator of water quality, some of the extracellular organic compounds contributing to TOC may be metabolised by micro-organisms, contributing to changes in virus integrity. To determine the quantity of organically

bound carbon, the organic molecules must be broken down into single carbon units which can be measured quantitatively. The method was carried out as per Standard Method 5310, 23rd edition.

This method measured TOC by non-purgeable organic carbon (NPOC). The sample was acidified and sparged, then introduced via a sample injector into a combustion tube filled with an oxidation catalyst (Pd/Pt beads) heated to 750°C. Carbon in the sample was first converted to carbon dioxide (CO₂) by the combustion furnace for TOC analysis. A carrier gas swept the derived CO₂ through a non-dispersive infrared (NDIR) detector. The NDIR generated a signal that was proportional to the concentration of CO₂ in the carrier gas. That signal was then linearised and integrated over the sample analysis time. The resulting area was then compared to the calibration data and a sample concentration in milligrams per litre (mg/L) was calculated.

Suspended solids

Solid analyses are important in the control of biological and physical wastewater treatment processes and (along with turbidity, which is frequently correlated) in understanding the physical nature of the environmental samples collected. Suspended solids or total suspended matter is the retained material (measured gravimetrically) on a standard glass fibre filter (with a nominal pore size of 0.5 µm or smaller) after filtration of a well-mixed sample under vacuum. The residue was dried at 105°C ± 2°C. The chemical and physical nature of the material in suspension, the pore size of the filter, the area and thickness of the filter mat and the amount and physical state of the materials deposited on the filter are the principal factors affecting the separation of suspended solids from dissolved solids. The method was carried out using Standard Method 2540, 23rd edition. Results were reported in mg/L using the formula below:

$$\text{Suspended solids (mg/L)} = \frac{[(M2 - M1)_{\text{sample}} - \text{Blank}] \times 1000 \text{ mg/g} \times 1000 \text{ ml/L}}{V}$$

Where M1 = mass of filter paper, in grams (g)

M2 = mass of filter and dried residue, in grams (g)

V = volume of sample aliquot chosen, in millilitres (ml)

Chemical Oxygen Demand (COD)

The COD of a sample is a measure of the oxygen equivalent of the organic matter content that is susceptible to oxidation by a strong chemical oxidant (presuming that the inorganic compounds present are most likely in an oxidised state in environmental waters). This method used potassium dichromate as the oxidant and this gives 90 - 100% of the theoretical oxidation value for most organic compounds. These data are useful independently as an assessment of a compound of the environmental water quality, but also in conjunction with and verification of BOD and TOC result behaviour in the samples.

The sample was preserved by the addition of 1 mL concentrated sulphuric acid per 100 mL of sample. The sample was agitated and an appropriate amount of a sample was transferred into a COD tube pre-packed with reagents. The vial was then heated for two hours at approximately 148°C. Once digestion was completed, samples were cooled to room temperature and results were obtained using a photometer and reported as mg O₂/L. The method was carried out as per ISO 15705.

Conductivity

The conductivity of an aqueous solution is a numerical expression of its ability to conduct an electric current. This ability depends on the ions in solution, their concentrations (total and relative), mobility, valence and temperature of measurement. Compounds that dissociate readily in solution, usually inorganic, ionise giving conductive solutions. The measurement made is usually resistance, which is the reciprocal of conductivity.

The measurement of conductivity of the sample thus allows evaluation of the degree of dissolved substances in the water (including from contamination sources such as sewer spills) and is a measurement of the daily variation over time at a particular sampling point and between different sample points. The conductivity of a solution is affected by the temperature hence in this method, all measurements were temperature compensated. The assigned

compensation temperature is 25°C. Conductivity was measured in units of micromhos/centimeter [$\mu\text{mho}/\text{cm}$] or, in SI units, millisiemens/metre [mS/m], where $1 \text{ mS}/\text{m} = 10 \mu\text{mhos}/\text{cm}$ using a WTW conductivity meter, Cond 7310. The method was carried out according to Standard Method 2510, 23rd edition.

Alkalinity

The alkalinity of a sample of water is its quantitative capacity to neutralize a strong acid to a specific pH or endpoint. Alkalinity measurements are used in the interpretation and control of water and wastewater treatment processes. In natural or treated water, alkalinity is usually due to the presence of bicarbonates, carbonates and hydroxides of calcium, magnesium, sodium and potassium, although measured values may include contributions from borates, phosphates and silicates. This also gives an idea of the pH buffering capacity of natural waters which may affect viral persistence. Calcium compounds are the main cause of alkalinity in natural water whereas industrial waste may have a complex mixture of contributory compounds. It is usual to express alkalinity as an equivalent amount of calcium carbonate.

Samples were collected in either plastic or glass containers and analysed within three days. No preservation was required and samples were stable at laboratory temperature. Approximately 50 ml aliquots of the sample(s) and/or AQC(s) were transferred into plastic sample cups and placed on the sample rack. A Mettler alkalinity auto-titrator with a pH electrode and a sample changer was used. The pH electrode was calibrated with buffers 4 and 7 before use. Results were automatically calculated by the auto-titrator's software. If samples were diluted, the results were multiplied by the dilution factor. Sample alkalinity was reported as mg/L CaCO_3 . The method was carried out as per Standard Method 2320, 23rd edition.

Ammonia

In water, the forms of nitrogen of greatest interest are organic nitrogen, nitrite, nitrate and ammonia due to their impact on eutrophication. All these forms of nitrogen, as well as nitrogen

gas, are biochemically interconvertible and involve other components of the nitrogen cycle (i.e., ammonification, nitrification and denitrification). Organic nitrogen is defined as organically bound nitrogen. Organic nitrogen and ammonia can be determined together known as Kjeldahl nitrogen. Total oxidised nitrogen (TON) is the sum of nitrite and nitrate. In the study area, the presence of detectable ammonia is considered an indicator of anthropogenic input, and as the area is not agricultural in character, this is primarily from sewage input(s).

Samples were collected in either glass or plastic containers and analysed as soon as possible. For preservation purposes, samples were stored at temperatures between 0°C to 10°C for up to 7 days. The method used was for the determination of ammonia based on the modified Berthelot reaction using the Aquakem 250 / 600 / Gallery Plus Discrete Analyser. Samples were filtered through a 0.45 µm membrane filter before analysis. Ammonia reacted with hypochlorite ions generated by the alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine. The monochloramine reacted with salicylate ions in the presence of sodium nitroprusside at approximately pH 12.6 to form 5-aminosalicylate. After oxidation and oxidative coupling, a blue-coloured complex was formed. The absorbance of the coloured complex was measured spectrophotometrically at a wavelength of 660 nm and was related to the ammonia concentration using a calibration curve (Beer Lambert's Law). The ammonia concentration was expressed in units of mg N/L. The method was carried out using Standard Method 4500, 23rd edition.

Total phosphate and orthophosphate (or soluble reactive phosphate, SRP)

Phosphorus occurs in natural water and wastewater almost solely as phosphates. In water, phosphorus exists solely in three basic forms, orthophosphates (H_3PO_4 , H_2PO_4^- , HPO_4^{2-} , PO_4^{3-}), (called SRP in the data utilised here), condensed phosphates (pyro-, meta- and polyphosphates) and organically bound phosphate. Orthophosphate is the only phosphorus that plants and micro-organisms can use directly. Phosphates in natural waters may also be present adsorbed to clays and in living tissue. While orthophosphate is of particular interest to assess water quality for eutrophication potential due to its immediate availability, it may not

persist in that form. The total amount of phosphate is the also of particular interest in assessing anthropogenic spatial and temporal water quality change, including from sewer spills or treated effluent. This is a modified standard method (4500) and the form of phosphate determined by this method is termed total phosphate (TP). Samples were collected in either glass or plastic containers. Soluble phosphate can be measured directly after suitable filtration by the method below. For total phosphate, other forms (condensed/inorganic phosphate and organic phosphate) needed to be converted into orthophosphate before analysis. This was achieved by the hydrolysis of the condensed phosphates in the presence of boiling sulphuric acid and the oxidation of the organic phosphates by persulphate in an autoclave. After the samples were digested in an autoclave, the soluble orthophosphate ion reacted with ammonium molybdate and antimony potassium tartrate (catalyst) under acidic conditions to form a 12-molybdophosphoric acid complex. The complex was then reduced with ascorbic acid (reducing agent) to form a blue hetero-poly compound. The absorbance of this compound was measured spectrophotometrically at a wavelength of 880 nm and was related to the phosphate concentration using a calibration curve (Beer-Lambert Law) using the Aquakem 250/600/Gallery Plus Discrete Analyser. The concentration of the SRP or total phosphate was expressed in units of $\mu\text{g P/L}$ or mg P/L . The method was carried out as per Standard Method 4500-P, 23rd edition.

Aluminium

The effects of metals in water range from beneficial and troublesome, to dangerously toxic. Metals may be determined satisfactorily by atomic absorption spectroscopy, inductively coupled plasma spectroscopy (ICP) or colorimetric methods. Inductively coupled plasma techniques are applicable over a broad linear range and are especially sensitive to refractory elements. In natural waters, aluminum data can potentially provide information on catchment changes and erosion/particulate effects potentially affecting virus viability. In this method aluminum (Al) was determined by measuring the emission signal using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). This method was adapted from Standard

Methods 3120B and 3030, 23rd edition. Samples were collected in glass or plastic bottles with 2 ml nitric acid per 100 ml of sample and reported in $\mu\text{g/L}$.

6.6 Appendix F Correlation matrices for sample sites

Table 13. Correlation matrix between abiotic factors and enteric viruses present in sample Site 1

| | % NH3 | Al | Alkalinity | BOD | Ca | Cl | PFU | Colour | mS/m | E.coli | Fe | Mg | Mn | Na | NH ₃ | NO ₃ | pH | Si | SO ₄ | SRP | SS | °C | TOC | TP | NTU | Adeno 40/41 | Adeno | Hep A | Rota A | Noro GI | Noro GII | Rainfall | | | | |
|-----------------|--------|--------|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|-----------------|-----------------|--------|--------|-----------------|--------|--------|--------|--------|--------|--------|-------------|--------|--------|--------|---------|----------|----------|---|---|--|--|
| % NH3 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Al | 0,081 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Alkalinity | -0,529 | 0,312 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| BOD | 0,245 | -0,170 | -0,159 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ca | -0,555 | 0,404 | 0,934 | -0,158 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cl | -0,311 | 0,874 | 0,410 | -0,285 | 0,494 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| PFU | 0,104 | 0,633 | 0,023 | -0,115 | 0,004 | 0,705 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Colour | -0,203 | 0,679 | 0,618 | -0,007 | 0,548 | 0,822 | 0,723 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| mS/m | -0,526 | 0,430 | 0,935 | -0,272 | 0,921 | 0,492 | 0,320 | 0,520 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| E.coli | 0,499 | -0,035 | -0,098 | 0,101 | 0,038 | 0,179 | -0,179 | 0,314 | -0,394 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Fe | -0,072 | 0,893 | 0,345 | -0,006 | 0,447 | 0,938 | 0,664 | 0,831 | 0,373 | 0,471 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| Mg | -0,614 | 0,250 | 0,847 | -0,356 | 0,872 | 0,273 | -0,316 | 0,231 | 0,932 | -0,380 | 0,152 | 1 | | | | | | | | | | | | | | | | | | | | | | | | |
| Mn | 0,536 | -0,350 | -0,412 | -0,057 | -0,562 | -0,424 | 0,339 | -0,169 | -0,508 | 0,108 | -0,415 | -0,660 | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| Na | -0,154 | 0,311 | 0,818 | 0,081 | 0,882 | 0,246 | -0,191 | 0,437 | 0,744 | 0,348 | 0,337 | 0,683 | -0,324 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| NH ₃ | 0,999 | 0,063 | -0,545 | 0,262 | -0,576 | -0,329 | 0,075 | -0,222 | -0,542 | 0,481 | -0,089 | -0,620 | 0,523 | -0,179 | 1 | | | | | | | | | | | | | | | | | | | | | |
| NO ₃ | 0,446 | 0,043 | -0,174 | 0,046 | -0,018 | -0,416 | -0,358 | -0,601 | 0,118 | -0,128 | -0,295 | 0,180 | -0,138 | 0,226 | 0,447 | 1 | | | | | | | | | | | | | | | | | | | | |
| pH | 0,956 | 0,269 | -0,426 | 0,245 | -0,481 | -0,192 | 0,074 | -0,123 | -0,096 | -0,321 | 0,021 | -0,460 | 0,379 | -0,144 | 0,960 | 0,461 | 1 | | | | | | | | | | | | | | | | | | | |
| Si | 0,649 | -0,221 | -0,659 | 0,509 | -0,508 | -0,502 | -0,470 | -0,625 | -0,630 | 0,425 | -0,253 | -0,448 | 0,008 | -0,203 | 0,665 | 0,683 | 0,572 | 1 | | | | | | | | | | | | | | | | | | |
| SO ₄ | -0,472 | 0,644 | 0,201 | -0,469 | 0,206 | 0,845 | 0,664 | 0,583 | 0,338 | -0,282 | 0,657 | 0,195 | -0,390 | -0,194 | -0,474 | -0,550 | -0,307 | -0,590 | 1 | | | | | | | | | | | | | | | | | |
| SRP | -0,151 | -0,079 | 0,120 | -0,188 | 0,373 | -0,118 | -0,359 | -0,455 | -0,166 | 0,812 | -0,151 | 0,530 | -0,424 | 0,379 | -0,161 | 0,192 | -0,468 | 0,289 | -0,233 | 1 | | | | | | | | | | | | | | | | |
| SS | 0,342 | 0,151 | -0,187 | 0,292 | -0,143 | 0,437 | 0,344 | 0,556 | -0,592 | 0,609 | 0,610 | -0,586 | 0,269 | -0,013 | 0,324 | -0,281 | 0,009 | 0,058 | 0,160 | 0,203 | 1 | | | | | | | | | | | | | | | |
| °C | 0,623 | -0,048 | -0,486 | 0,345 | -0,636 | -0,025 | 0,176 | 0,219 | -0,732 | 0,415 | 0,141 | -0,829 | 0,558 | -0,447 | 0,631 | -0,410 | 0,143 | 0,206 | -0,029 | -0,002 | 0,689 | 1 | | | | | | | | | | | | | | |
| TOC | -0,276 | 0,704 | 0,426 | -0,176 | 0,386 | 0,899 | 0,829 | 0,900 | 0,300 | 0,121 | 0,795 | 0,128 | -0,063 | 0,161 | -0,298 | -0,534 | -0,098 | -0,727 | 0,798 | -0,121 | 0,384 | 0,112 | 1 | | | | | | | | | | | | | |
| TP | 0,500 | 0,174 | -0,048 | 0,019 | -0,109 | 0,128 | 0,687 | 0,326 | -0,399 | 0,312 | 0,162 | -0,396 | 0,781 | 0,107 | 0,465 | -0,151 | 0,253 | -0,194 | -0,100 | -0,023 | 0,822 | 0,500 | 0,421 | 1 | | | | | | | | | | | | |
| NTU | 0,382 | 0,293 | -0,047 | 0,220 | 0,015 | 0,605 | 0,562 | 0,671 | -0,481 | 0,518 | 0,772 | -0,413 | 0,169 | 0,137 | 0,359 | -0,233 | 0,118 | 0,005 | 0,267 | 0,122 | 0,972 | 0,598 | 0,485 | 0,877 | 1 | | | | | | | | | | | |
| Adeno 40/41 | -0,859 | -0,239 | 0,821 | -0,201 | 0,848 | 0,602 | -0,360 | 0,797 | -0,212 | -0,322 | 0,922 | 0,973 | -0,853 | 0,570 | -0,872 | -0,540 | -0,001 | -0,831 | 0,175 | -0,212 | -0,261 | 0,208 | -0,461 | -0,262 | -0,239 | 1 | | | | | | | | | | |
| Adeno | -0,034 | -0,216 | -0,896 | -0,208 | -0,874 | 0,409 | -0,336 | -0,914 | -0,206 | -0,323 | -0,106 | -0,673 | -0,046 | -0,995 | -0,009 | -0,552 | -0,001 | 0,888 | 0,778 | -0,217 | -0,261 | 0,204 | -0,438 | -0,257 | -0,235 | 1,000 | 1 | | | | | | | | | |
| Hep A | -0,888 | 0,382 | -0,053 | -0,084 | -0,005 | 0,996 | 0,167 | -0,094 | -0,231 | 0,368 | 0,814 | 0,314 | -0,893 | -0,399 | -0,876 | -0,536 | -0,798 | 0,036 | 0,929 | 0,723 | 0,358 | 0,229 | 0,246 | 0,022 | 0,345 | -0,008 | -0,002 | 1 | | | | | | | | |
| Rota A | -0,975 | 0,445 | 0,200 | -0,008 | 0,246 | 0,987 | -0,178 | 0,159 | 0,192 | 0,119 | 0,934 | 0,543 | -0,977 | -0,156 | -0,969 | -0,174 | -0,437 | -0,216 | 0,807 | 0,288 | 0,091 | -0,383 | 0,064 | -0,160 | 0,131 | -0,076 | -0,076 | 0,631 | 1 | | | | | | | |
| Noro GI | -1 | -0,174 | -1 | - | -1 | 1 | -1 | -1 | -0,329 | -0,463 | 1 | 1 | -1 | -1 | -1 | -0,722 | -0,501 | 1 | 1 | - | -0,757 | 0,304 | -0,999 | -0,409 | -0,528 | 1,000 | 1,000 | 0,484 | 0,521 | 1 | | | | | | |
| Noro GII | 1 | -0,768 | 1 | - | 1 | -1 | 1 | 1 | 0,983 | 0,999 | -1 | -1 | 1 | 1 | 1 | 0,960 | 1,000 | -1 | -1 | - | 0,943 | 0,675 | 0,532 | 0,995 | 0,999 | -0,498 | -0,496 | -1,000 | -1,000 | -0,497 | 1 | | | | | |
| Rainfall | 0,364 | -0,173 | -0,421 | 0,169 | -0,331 | -0,228 | -0,299 | -0,090 | -0,300 | 0,896 | 0,049 | -0,589 | 0,075 | -0,078 | 0,372 | -0,131 | -0,343 | 0,630 | -0,428 | 0,779 | 0,278 | 0,288 | -0,045 | -0,064 | 0,158 | - | - | - | - | - | - | - | - | 1 | | |

Table 14. Correlation matrix between abiotic factors and enteric viruses present in sample Site 2

| | % NH3 | Al | Alkalinity | BOD | Ca | Cl | COD | PFU | mS/m | DO | E. coli | F | K | Mg | Na | NH ₃ | NH ₃ (F) | NO ₃ | pH | Si | SO ₄ | SRP | SS | °C | TOC | TP | NTU | Adeno 40/41 | Adeno | Hep A | Rota A | Noro GI | Noro GII | Rainfall | | |
|---------------------|--------|--------|------------|--------|--------|--------|--------|--------|--------|--------|----------|--------|--------|--------|--------|-----------------|---------------------|-----------------|--------|--------|-----------------|--------|--------|--------|--------|--------|--------|-------------|--------|--------|--------|----------|----------|----------|--|--|
| % NH3 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Al | 0,099 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Alkalinity | -0,163 | 0,380 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| BOD | -0,203 | 0,113 | 0,736 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ca | -0,280 | 0,325 | 0,509 | 0,275 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cl | -0,139 | 0,427 | 0,973 | 0,662 | 0,554 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| COD | -0,242 | -0,001 | 0,039 | -0,124 | -0,043 | -0,034 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| PFU | -0,079 | 0,397 | 0,959 | 0,663 | 0,528 | 0,993 | -0,094 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| mS/m | -0,138 | 0,364 | 0,983 | 0,670 | 0,551 | 0,994 | -0,033 | 0,990 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| DO | 0,360 | -0,369 | 0,027 | -0,060 | -0,267 | 0,115 | -0,160 | 0,195 | 0,115 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| E. coli | -0,117 | 0,363 | 0,956 | 0,669 | 0,540 | 0,994 | -0,078 | 1,000 | 0,988 | 0,173 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| F | -0,119 | 0,365 | 0,957 | 0,669 | 0,541 | 0,994 | -0,077 | 1,000 | 0,988 | 0,171 | 0,999991 | 1 | | | | | | | | | | | | | | | | | | | | | | | | |
| K | -0,204 | 0,078 | -0,116 | -0,124 | 0,785 | -0,071 | -0,077 | -0,094 | -0,076 | -0,350 | -0,078 | -0,077 | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| Mg | -0,238 | 0,392 | 0,767 | 0,453 | 0,530 | 0,764 | 0,010 | 0,742 | 0,764 | 0,030 | 0,745 | 0,747 | 0,065 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| Na | -0,230 | 0,146 | 0,090 | 0,014 | 0,897 | 0,139 | -0,085 | 0,114 | 0,135 | -0,315 | 0,133 | 0,134 | 0,977 | 0,228 | 1 | | | | | | | | | | | | | | | | | | | | | |
| NH ₃ | -0,055 | 0,333 | 0,941 | 0,646 | 0,516 | 0,980 | -0,097 | 0,995 | 0,977 | 0,184 | 0,992 | 0,992 | -0,097 | 0,714 | 0,111 | 1 | | | | | | | | | | | | | | | | | | | | |
| NH ₃ (F) | 0,568 | 0,267 | 0,657 | 0,385 | 0,243 | 0,699 | -0,230 | 0,765 | 0,703 | 0,367 | 0,727 | 0,726 | -0,205 | 0,417 | -0,052 | 0,783 | 1 | | | | | | | | | | | | | | | | | | | |
| NO ₃ | -0,192 | 0,278 | 0,832 | 0,674 | 0,504 | 0,897 | -0,201 | 0,921 | 0,880 | 0,286 | 0,915 | 0,916 | -0,024 | 0,795 | 0,166 | 0,882 | 0,571 | 1 | | | | | | | | | | | | | | | | | | |
| pH | 0,782 | 0,063 | -0,290 | -0,278 | -0,311 | -0,271 | -0,256 | -0,234 | -0,272 | 0,450 | -0,255 | -0,256 | -0,122 | -0,187 | -0,185 | -0,211 | 0,292 | -0,257 | 1 | | | | | | | | | | | | | | | | | |
| Si | -0,046 | 0,042 | 0,542 | 0,524 | 0,440 | 0,635 | -0,405 | 0,717 | 0,612 | 0,345 | 0,697 | 0,696 | 0,119 | 0,606 | 0,253 | 0,717 | 0,569 | 0,791 | -0,034 | 1 | | | | | | | | | | | | | | | | |
| SO ₄ | -0,103 | 0,393 | 0,959 | 0,663 | 0,545 | 0,995 | -0,070 | 0,999 | 0,989 | 0,139 | 0,998 | 0,998 | -0,075 | 0,744 | 0,135 | 0,993 | 0,736 | 0,902 | -0,247 | 0,678 | 1 | | | | | | | | | | | | | | | |
| SRP | -0,099 | 0,416 | 0,902 | 0,627 | 0,499 | 0,955 | -0,098 | 0,999 | 0,939 | 0,073 | 0,967 | 0,967 | -0,098 | 0,700 | 0,101 | 0,976 | 0,733 | 0,861 | -0,248 | 0,721 | 0,974 | 1 | | | | | | | | | | | | | | |
| SS | 0,231 | 0,164 | 0,246 | 0,285 | 0,044 | 0,353 | -0,136 | 0,436 | 0,299 | 0,462 | 0,392 | 0,390 | -0,136 | 0,025 | -0,066 | 0,380 | 0,425 | 0,441 | 0,006 | 0,434 | 0,364 | 0,347 | 1 | | | | | | | | | | | | | |
| °C | 0,576 | 0,095 | 0,081 | 0,038 | -0,200 | 0,185 | -0,261 | 0,306 | 0,151 | 0,500 | 0,244 | 0,241 | -0,324 | -0,218 | -0,289 | 0,301 | 0,603 | 0,125 | 0,496 | 0,335 | 0,244 | 0,326 | 0,563 | 1 | | | | | | | | | | | | |
| TOC | 0,331 | 0,591 | 0,681 | 0,175 | 0,410 | 0,708 | 0,065 | 0,693 | 0,704 | -0,035 | 0,677 | 0,676 | -0,065 | 0,482 | 0,087 | 0,698 | 0,769 | 0,454 | -0,039 | 0,201 | 0,699 | 0,666 | 0,255 | 0,381 | 0,610 | 1 | | | | | | | | | | |
| TP | -0,097 | 0,415 | 0,834 | 0,556 | 0,443 | 0,892 | 0,054 | 0,973 | 0,865 | 0,081 | 0,908 | 0,908 | -0,122 | 0,676 | 0,063 | 0,918 | 0,685 | 0,801 | -0,224 | 0,702 | 0,916 | 0,967 | 0,345 | 0,381 | 0,610 | 1 | | | | | | | | | | |
| NTU | 0,298 | 0,266 | -0,102 | -0,010 | -0,129 | 0,030 | -0,178 | 0,119 | -0,051 | 0,198 | 0,076 | 0,074 | -0,122 | -0,120 | -0,122 | 0,080 | 0,221 | 0,149 | 0,110 | 0,328 | 0,068 | 0,174 | 0,716 | 0,644 | 0,054 | 0,288 | 1 | | | | | | | | | |
| Adeno 40/41 | -0,115 | 0,228 | -0,066 | 0,335 | -0,151 | -0,135 | 0,287 | -0,103 | -0,151 | -0,739 | -0,158 | -0,157 | - | -0,048 | -0,175 | -0,194 | -0,259 | -0,046 | -0,429 | -0,253 | -0,142 | -0,113 | -0,034 | -0,575 | -0,118 | -0,086 | 0,123 | 1 | | | | | | | | |
| Adeno | 0,256 | 0,394 | -0,258 | -0,224 | -0,166 | -0,171 | -0,141 | -0,198 | -0,221 | -0,581 | -0,145 | -0,145 | - | -0,174 | -0,208 | -0,086 | 0,067 | -0,191 | 0,070 | 0,125 | -0,112 | 0,110 | -0,222 | 0,460 | 0,038 | 0,220 | 0,862 | 0,231 | 1 | | | | | | | |
| Hep A | -0,249 | 0,377 | 0,161 | -0,183 | 0,288 | 0,196 | 0,334 | 0,342 | 0,176 | -0,464 | 0,196 | 0,196 | - | 0,270 | 0,209 | 0,210 | 0,075 | 0,061 | -0,458 | 0,068 | 0,221 | 0,353 | -0,301 | 0,215 | 0,292 | 0,517 | 0,541 | -0,044 | 0,607 | 1 | | | | | | |
| Rota A | -0,232 | -0,268 | 0,307 | 0,077 | 0,257 | 0,217 | 0,575 | 0,054 | 0,252 | 0,483 | 0,178 | 0,178 | - | 0,252 | 0,263 | 0,136 | -0,003 | 0,113 | -0,122 | -0,190 | 0,156 | -0,033 | 0,091 | -0,373 | 0,145 | -0,020 | -0,723 | -0,263 | -0,830 | -0,205 | 1 | | | | | |
| Noro GI | -0,830 | 0,839 | -0,853 | - | -0,602 | 0,947 | - | -1,000 | -0,939 | 0,172 | -0,913 | - | - | 0,807 | -0,893 | -0,998 | -0,924 | 0,891 | -0,767 | -0,714 | -0,935 | 0,767 | 0,172 | -0,420 | -0,785 | 0,394 | 0,841 | 0,168 | -0,914 | -0,011 | -0,618 | 1 | | | | |
| Noro GII | 0,959 | -0,637 | 0,657 | - | 0,335 | -0,808 | - | -1,000 | 0,793 | 0,131 | 0,749 | - | - | -0,947 | 0,718 | 0,970 | 0,996 | -0,714 | 0,924 | 0,471 | 0,998 | -0,924 | 0,131 | 0,129 | 0,934 | -0,651 | -0,640 | 0,135 | 0,751 | -0,289 | 0,354 | -0,95406 | 1 | | | |
| Rainfall | 0,266 | -0,120 | 0,355 | 0,286 | 0,143 | 0,445 | -0,131 | 0,502 | 0,444 | 0,584 | 0,493 | 0,493 | -0,131 | 0,220 | -0,025 | 0,485 | 0,531 | 0,579 | 0,094 | 0,427 | 0,487 | 0,450 | 0,324 | 0,413 | 0,251 | 0,460 | 0,232 | -0,157 | -0,145 | 0,196 | 0,178 | - | - | 1 | | |

Table 15. Correlation matrix between abiotic factors and enteric viruses present in sample Site 3

| | Al | BOD | COD | PFU | mS/m | E. coli | NH ₃ | NO ₃ | pH | SRP | SS | °c | TOC | TP | NTU | Adeno 40/41 | Adeno | Hep A | Rota A | Noro GI | Noro GII | Rainfall | | |
|-----------------|----------|----------|----------|----------|----------|----------|-----------------|-----------------|----------|----------|----------|----------|----------|----------|----------|-------------|----------|----------|----------|----------|----------|----------|--|--|
| Al (AE) | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| BOD | -0,26703 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| COD | -0,14521 | 0,048793 | 1 | | | | | | | | | | | | | | | | | | | | | |
| PFU | -0,02999 | -0,26486 | -0,12724 | 1 | | | | | | | | | | | | | | | | | | | | |
| mS/m | -0,09013 | 0,506499 | 0,105471 | 0,005845 | 1 | | | | | | | | | | | | | | | | | | | |
| E. coli | -0,29478 | -0,12227 | 0,231889 | 0,677216 | 0,102695 | 1 | | | | | | | | | | | | | | | | | | |
| NH ₃ | -0,43867 | 0,363512 | 0,547823 | 0,178472 | 0,390415 | 0,410235 | 1 | | | | | | | | | | | | | | | | | |
| NO ₃ | 0,035238 | -0,22475 | -0,19686 | -0,12016 | 0,416289 | 0,054158 | -0,39483 | 1 | | | | | | | | | | | | | | | | |
| pH OS | -0,27932 | 0,188507 | -0,21999 | -0,18364 | 0,223473 | -0,02278 | -0,12354 | 0,233844 | 1 | | | | | | | | | | | | | | | |
| SRP | -0,33472 | 0,262709 | 0,455132 | 0,10037 | 0,405296 | 0,408084 | 0,740016 | 0,076348 | -0,06239 | 1 | | | | | | | | | | | | | | |
| SS | 0,276928 | 0,283513 | -0,04342 | 0,031003 | -0,072 | 0,060663 | 0,304264 | -0,65379 | -0,30137 | -0,13173 | 1 | | | | | | | | | | | | | |
| °c | -0,02104 | -0,21707 | -0,60716 | 0,095471 | -0,05068 | 0,071198 | -0,59255 | 0,625486 | 0,464944 | -0,23889 | -0,51012 | 1 | | | | | | | | | | | | |
| TOC | -0,36171 | 0,401182 | 0,570222 | -0,09786 | 0,202921 | 0,270376 | 0,821609 | -0,50458 | 0,013814 | 0,488458 | 0,485417 | -0,6619 | 1 | | | | | | | | | | | |
| TP | -0,38379 | 0,191501 | 0,582216 | -0,24503 | -0,1933 | -0,12293 | 0,596248 | -0,67321 | -0,15052 | 0,225968 | 0,16598 | -0,65187 | 0,575005 | 1 | | | | | | | | | | |
| NTU | 0,602921 | 0,316877 | -0,04029 | -0,04972 | 0,046373 | -0,07061 | 0,100087 | -0,42296 | -0,37036 | -0,04275 | 0,829859 | -0,47785 | 0,262898 | -0,07214 | 1 | | | | | | | | | |
| Adeno 40/41 | 0,346045 | 0,340642 | -0,1096 | -0,45684 | -0,11518 | -0,28248 | -0,05407 | -0,40598 | 0,062758 | -0,34271 | 0,758595 | -0,27608 | 0,266539 | 0,154458 | 0,651911 | 1 | | | | | | | | |
| Adeno | -0,05213 | 0,206817 | -0,1161 | 0,133075 | 0,015233 | -0,15605 | -0,28512 | -0,04043 | 0,079458 | -0,21907 | -0,08395 | -0,06222 | -0,12308 | -0,28608 | 0,105249 | -0,26813 | 1 | | | | | | | |
| Hep A | -0,06473 | -0,24691 | 0,11608 | 0,000906 | 0,208651 | -0,16646 | 0,045013 | 0,081299 | 0,034799 | -0,01986 | -0,10532 | -0,38866 | 0,135821 | -0,04158 | -0,05712 | -0,25788 | 0,495994 | 1 | | | | | | |
| Rota A | 0,149642 | 0,028755 | -0,09046 | 0,522841 | 0,063139 | 0,353683 | -0,08569 | 0,058651 | 0,374483 | 0,036168 | -0,18935 | 0,273854 | -0,13034 | -0,22685 | -0,0992 | 0,067324 | -0,12305 | -0,2503 | 1 | | | | | |
| Noro GI | -0,74485 | 0,33934 | -0,15311 | -0,71871 | 0,128699 | 0,03883 | 0,190867 | 0,423243 | -0,17963 | 0,173191 | 0,665924 | 0,065845 | 0,355376 | -0,00422 | 0,323739 | 0,049147 | 0,068458 | -0,14708 | -0,85325 | 1 | | | | |
| Noro GII | 0,083952 | 0,082794 | -0,22246 | 0,226024 | 0,175953 | -0,05325 | -0,22874 | 0,040432 | -0,36837 | -0,23815 | 0,854547 | -0,34902 | -0,14188 | -0,48395 | 0,497446 | -0,66837 | 0,950047 | 0,747739 | -0,47069 | 0,344391 | 1 | | | |
| Rainfall | 0,102001 | 0,14043 | -0,14546 | 0,407453 | 0,121834 | -0,00563 | -0,22856 | 0,188223 | 0,393583 | -0,07769 | -0,19017 | 0,399628 | -0,24826 | -0,40684 | -0,05941 | 0,000112 | 0,191432 | -0,32445 | 0,7951 | -0,54149 | 0,062037 | 1 | | |

Table 16. Correlation matrix between abiotic factors and enteric viruses present in sample Site 4

| | AI | BOD | COD | PFU | mS/m | E. coli | NH ₃ | NO ₃ | pH | SRP | SS | °C | TOC | TP | NTU | Adeno 40/41 | Adeno | Hep A | Rota A | Noro GI | Noro GII | Rainfall | | |
|-----------------|----------|----------|----------|----------|----------|----------|-----------------|-----------------|----------|----------|----------|----------|----------|----------|----------|-------------|----------|----------|----------|----------|----------|----------|--|--|
| AI | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| BOD | -0,30076 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| COD | -0,21878 | 0,110448 | 1 | | | | | | | | | | | | | | | | | | | | | |
| PFU | -0,1281 | 0,09208 | 0,308558 | 1 | | | | | | | | | | | | | | | | | | | | |
| mS/m | -0,22348 | 0,168538 | 0,031234 | -0,04993 | 1 | | | | | | | | | | | | | | | | | | | |
| E. coli | 0,216054 | -0,03195 | -0,15254 | 0,096252 | -0,1045 | 1 | | | | | | | | | | | | | | | | | | |
| NH ₃ | -0,20587 | 0,663051 | 0,242328 | 0,282544 | 0,603372 | 0,125486 | 1 | | | | | | | | | | | | | | | | | |
| NO ₃ | 0,34507 | -0,85137 | -0,2252 | -0,3608 | -0,25202 | 0,069537 | -0,69233 | 1 | | | | | | | | | | | | | | | | |
| pH | -0,36091 | 0,168294 | 0,472709 | 0,790527 | 0,382652 | -0,2701 | 0,775101 | -0,77139 | 1 | | | | | | | | | | | | | | | |
| SRP | -0,376 | 0,459144 | 0,063924 | 0,249704 | 0,052914 | 0,342618 | 0,632339 | -0,44047 | 0,810837 | 1 | | | | | | | | | | | | | | |
| SS | -0,09387 | 0,468863 | 0,031423 | -0,21855 | 0,160098 | -0,58288 | 0,215016 | -0,35359 | 0,150756 | -0,21574 | 1 | | | | | | | | | | | | | |
| °C | -0,37572 | -0,54554 | 0,658542 | -0,02868 | -0,942 | 0,010902 | -0,74386 | 0,666767 | 0,005224 | 0,01362 | -0,98281 | 1 | | | | | | | | | | | | |
| TOC | -0,25575 | 0,645914 | -0,01666 | 0,275207 | 0,530276 | -0,05298 | 0,787164 | -0,60011 | 0,466297 | 0,497956 | 0,481885 | -0,90575 | 1 | | | | | | | | | | | |
| TP | -0,3721 | 0,557269 | 0,283127 | 0,514894 | 0,488272 | -0,18913 | 0,781035 | -0,74637 | 0,934302 | 0,622289 | 0,147445 | -0,51031 | 0,74303 | 1 | | | | | | | | | | |
| NTU | 0,519179 | 0,344426 | -0,48842 | 0,129632 | 0,988425 | 0,086061 | 0,799953 | -0,62179 | -0,1301 | -0,07933 | 0,910463 | -0,90775 | 0,838365 | 0,568051 | 1 | | | | | | | | | |
| Adeno 40/41 | 0,138676 | 0,496319 | -0,10152 | 0,152302 | 0,356481 | 0,274471 | 0,559027 | -0,55871 | 0,281593 | 0,009583 | 0,069842 | -0,64581 | 0,267073 | 0,13694 | 0,602375 | 1 | | | | | | | | |
| Adeno | -0,01171 | 0,435244 | -0,23507 | 0,067666 | -0,17703 | 0,006777 | 0,000978 | -0,23959 | 0,537744 | 0,019628 | 0,277869 | 0,036133 | 0,264264 | -0,03722 | -0,20233 | 0,475197 | 1 | | | | | | | |
| Hep A | -0,237 | 0,182624 | -0,10203 | 0,001925 | -0,1138 | 0,278182 | 0,413692 | -0,16643 | 0,770041 | 0,897805 | -0,22145 | 0,516197 | 0,30136 | 0,427247 | -0,75001 | -0,21872 | -0,22175 | 1 | | | | | | |
| Rota A | -0,29727 | -0,14967 | -0,10184 | 0,601943 | 0,034094 | -0,27314 | -0,22443 | -0,12162 | 0,806497 | -0,05229 | -0,22161 | 0,394416 | 0,045714 | 0,415347 | -0,62453 | -0,27416 | -0,17678 | -0,06909 | 1 | | | | | |
| Noro GI | 0,398874 | 0,904205 | 0,167445 | -0,51064 | -0,18463 | 0,196237 | 0,59245 | -0,46421 | - | 0,554757 | -0,23805 | - | 0,43534 | 0,381532 | - | -0,27751 | 0,03242 | 0,601619 | -0,29448 | 1 | | | | |
| Noro GII | 0,279061 | 0,775348 | -0,30708 | -0,23453 | -0,26387 | 0,53239 | 0,880884 | -0,59849 | - | 0,833174 | -0,51641 | - | 0,701032 | 0,449458 | - | -0,0874 | 0,119752 | 0,836916 | -0,42322 | 0,8194 | 1 | | | |
| Rainfall | 0,295015 | 0,238402 | -0,19942 | 0,078507 | 0,413957 | -0,07133 | 0,388986 | -0,35067 | -0,09129 | -0,1996 | 0,542714 | -0,88063 | 0,544722 | 0,245282 | 0,856876 | 0,193636 | -0,16988 | -0,11789 | 0,035778 | -0,26486 | -0,12705 | 1 | | |

Table 17. Correlation matrix between abiotic factors and enteric viruses present in sample Site 5

| | Al (AE) | BOD | PFU | mS/m | E. coli | NH ₃ | NO ₃ | pH | SRP | SS | °c | TOC | TP | NTU | Adeno 40/41 | Adeno | Hep A | Rota A | Noro GI | Noro GII | Rainfall | |
|-----------------|---------|----------|----------|----------|----------|-----------------|-----------------|----------|----------|----------|----------|----------|----------|----------|-------------|----------|----------|----------|----------|----------|----------|--|
| Al (AE) | 1 | | | | | | | | | | | | | | | | | | | | | |
| BOD | -0,200 | 1 | | | | | | | | | | | | | | | | | | | | |
| PFU | 0,744 | -0,10087 | 1 | | | | | | | | | | | | | | | | | | | |
| mS/m | -0,070 | 0,050997 | -0,05111 | 1 | | | | | | | | | | | | | | | | | | |
| E. coli | 0,559 | -0,04527 | 0,533671 | 0,013893 | 1 | | | | | | | | | | | | | | | | | |
| NH ₃ | 0,054 | -0,11479 | -0,14179 | 0,768678 | -0,04741 | 1 | | | | | | | | | | | | | | | | |
| NO ₃ | 0,154 | -0,05302 | 0,288547 | -0,44932 | 0,374844 | -0,7659 | 1 | | | | | | | | | | | | | | | |
| pH | 0,166 | -0,60883 | 0,255064 | -0,13223 | 0,036261 | -0,01083 | 0,196775 | 1 | | | | | | | | | | | | | | |
| SRP | -0,228 | -0,29659 | 0,224311 | -0,26106 | -0,04525 | -0,38132 | 0,323955 | 0,123044 | 1 | | | | | | | | | | | | | |
| SS | 0,553 | -0,11924 | 0,330967 | -0,0431 | 0,850859 | -0,03658 | 0,313283 | 0,313588 | -0,28774 | 1 | | | | | | | | | | | | |
| °c | -0,152 | -0,14963 | -0,59516 | -0,04484 | -0,30147 | 0,175967 | -0,20733 | 0,545419 | -0,233 | -0,22614 | 1 | | | | | | | | | | | |
| TOC | 0,490 | 0,114957 | 0,828891 | 0,335142 | 0,54859 | -0,01002 | 0,217838 | 0,008747 | 0,173923 | 0,340434 | -0,73652 | 1 | | | | | | | | | | |
| TP | 0,075 | -0,13828 | 0,214615 | -0,06151 | 0,364827 | -0,15085 | 0,290302 | -0,04318 | 0,116038 | 0,112972 | -0,47249 | 0,162787 | 1 | | | | | | | | | |
| NTU | 0,702 | -0,05411 | 0,46106 | -0,52386 | -0,23368 | -0,30002 | -0,06605 | 0,552 | -0,39033 | 0,577932 | 0,039887 | -0,28821 | -0,4246 | 1 | | | | | | | | |
| Adeno 40/41 | 0,209 | 0,31719 | 0,084963 | 0,015104 | 0,429095 | -0,03312 | -0,08796 | -0,547 | 0,028986 | 0,420878 | -0,29727 | 0,344917 | 0,079601 | -0,06584 | 1 | | | | | | | |
| Adeno | -0,339 | 0,345157 | 0,329445 | 0,456376 | 0,088593 | 0,073389 | -0,06948 | -0,02866 | 0,206408 | 0,040391 | -0,46063 | 0,714345 | -0,14029 | -0,17222 | 0,210304 | 1 | | | | | | |
| Hep A | 0,029 | -0,01457 | 0,044158 | -0,38201 | 0,253201 | -0,40288 | 0,23418 | -0,1021 | 0,186986 | 0,108503 | -0,15511 | -0,08986 | 0,653933 | -0,18079 | 0,282293 | -0,31399 | 1 | | | | | |
| Rota A | 0,372 | -0,06805 | 0,005217 | -0,01 | -0,12725 | 0,224916 | -0,23355 | 0,260487 | -0,24049 | 0,013658 | 0,439173 | -0,2188 | -0,12825 | 0,325132 | -0,06494 | -0,19184 | -0,19307 | 1 | | | | |
| Noro GI | -0,493 | 0,856126 | -0,95898 | 0,961213 | -0,9761 | 0,763112 | 0,760924 | 0,99993 | -0,43741 | 0,316932 | 0,942853 | 0,776125 | 0,206035 | -0,16916 | -0,6903 | 0,816634 | -0,14004 | -0,07006 | 1 | | | |
| Noro GII | -0,920 | 0,085659 | -0,67863 | 0,740794 | -0,36274 | 0,846929 | 0,762784 | 0,556361 | 0,478155 | -0,49216 | 0,786325 | 0,910103 | 0,844489 | -0,89086 | -0,39184 | 0,30915 | 0,4138 | 0,540157 | 0,546991 | 1 | | |
| Rainfall | -0,114 | -0,13681 | -0,34514 | -0,28195 | 0,061064 | -0,26692 | 0,156923 | -0,4219 | -0,23564 | 0,252258 | -0,3196 | -0,38271 | 0,205587 | 0,156747 | 0,197674 | -0,23843 | 0,247743 | -0,13862 | 0,169027 | -0,59935 | 1 | |

Table 18. Correlation matrix between abiotic factors and enteric viruses present in sample Site 6

| | AI | BOD | COD | PFU | mS/m | E. coli | NH ₃ | NO ₃ | pH | SRP | SS | °C | TOC | TP | NTU | Adeno 40/41 | Adeno | Hep A | Rota A | Noro GI | Noro GII | Rainfall | |
|-----------------|----------|----------|----------|----------|----------|----------|-----------------|-----------------|----------|----------|----------|----------|----------|----------|----------|-------------|----------|----------|----------|----------|----------|----------|--|
| AI | 1 | | | | | | | | | | | | | | | | | | | | | | |
| BOD | 0,275679 | 1 | | | | | | | | | | | | | | | | | | | | | |
| COD | -0,03002 | 0,592239 | 1 | | | | | | | | | | | | | | | | | | | | |
| PFU | 0,724669 | 0,202985 | -0,17268 | 1 | | | | | | | | | | | | | | | | | | | |
| mS/m | 0,051908 | 0,798174 | 0,615724 | 0,069587 | 1 | | | | | | | | | | | | | | | | | | |
| E. coli | 0,566685 | 0,247947 | -0,19702 | 0,5685 | -0,27837 | 1 | | | | | | | | | | | | | | | | | |
| NH ₃ | 0,066894 | 0,820062 | 0,902467 | -0,07592 | 0,692895 | -0,15942 | 1 | | | | | | | | | | | | | | | | |
| NO ₃ | 0,156529 | -0,49159 | -0,64087 | 0,315861 | -0,14795 | 0,081377 | -0,70021 | 1 | | | | | | | | | | | | | | | |
| pH | 0,096563 | 0,111141 | -0,28393 | 0,130691 | -0,14012 | 0,09006 | -0,15521 | 0,308643 | 1 | | | | | | | | | | | | | | |
| SRP | -0,26081 | -0,48839 | -0,2875 | 0,178586 | -0,08463 | -0,15591 | -0,38916 | 0,521038 | -0,37736 | 1 | | | | | | | | | | | | | |
| SS | 0,529878 | 0,401938 | -0,1474 | 0,306004 | -0,31877 | 0,767974 | -0,04448 | -0,18019 | 0,203582 | -0,52728 | 1 | | | | | | | | | | | | |
| °C | -0,18216 | -0,03855 | 0,100125 | -0,68085 | -0,35959 | -0,00047 | 0,11402 | -0,42236 | 0,115475 | -0,51902 | 0,470148 | 1 | | | | | | | | | | | |
| TOC | 0,520233 | 0,473523 | -0,02054 | 0,760575 | 0,398712 | 0,534891 | 0,124106 | 0,112195 | 0,029906 | -0,07553 | 0,325289 | -0,56357 | 1 | | | | | | | | | | |
| TP | 0,429964 | 0,151013 | -0,24245 | 0,810365 | 0,067416 | 0,519244 | -0,12534 | 0,316469 | 0,04951 | 0,324904 | 0,213053 | -0,60143 | 0,771882 | 1 | | | | | | | | | |
| NTU | 0,681947 | 0,250455 | -0,15881 | 0,374159 | -0,47036 | 0,761905 | -0,05924 | -0,2448 | 0,191027 | -0,53611 | 0,932618 | 0,463768 | 0,234751 | 0,233446 | 1 | | | | | | | | |
| Adeno 40/41 | 0,185307 | 0,076706 | 0,074602 | 0,094462 | 0,452846 | -0,22959 | -0,02244 | 0,331008 | -0,15661 | 0,183636 | -0,35472 | -0,5292 | 0,128056 | 0,113862 | -0,40594 | 1 | | | | | | | |
| Adeno | 0,12274 | 0,151985 | -0,00563 | -0,08341 | 0,32282 | -0,1471 | -0,02831 | 0,213384 | 0,23171 | -0,14686 | -0,30436 | -0,30781 | 0,075362 | -0,20801 | -0,37034 | 0,624105 | 1 | | | | | | |
| Hep A | -0,21164 | -0,08357 | -0,01522 | -0,27103 | 0,322905 | -0,51251 | -0,0276 | 0,258917 | 0,276731 | -0,11668 | -0,08601 | 0,2152 | -0,15351 | -0,19554 | -0,25871 | 0,249459 | -0,03249 | 1 | | | | | |
| Rota A | 0,136416 | -0,21823 | -0,07786 | -0,18711 | -0,23564 | -0,059 | -0,17884 | -0,05909 | -0,35565 | -0,14979 | 0,063377 | 0,052343 | -0,25589 | -0,31629 | 0,162873 | 0,248118 | -0,05648 | -0,03712 | 1 | | | | |
| Noro GI | 0,918979 | -0,74435 | - | 0,373041 | -0,83601 | 0,677885 | -0,59436 | -0,47961 | -0,11069 | -0,16314 | -0,42414 | -0,55083 | -0,97326 | -0,42176 | 0,435751 | -0,09318 | 0,500806 | -0,74956 | 0,477436 | 1 | | | |
| Noro GII | 0,965728 | -0,52313 | - | 0,301277 | -0,73715 | 0,283885 | -0,63975 | -0,73311 | -0,24858 | -0,46324 | 0,048449 | -0,35105 | -0,95458 | -0,77221 | 0,832666 | 0,411204 | 0,311957 | -0,46402 | 0,833638 | 0,861003 | 1 | | |
| Rainfall | -0,07233 | -0,31274 | -0,13599 | -0,35821 | -0,34931 | 0,202904 | -0,26044 | 0,015583 | -0,2739 | -0,13507 | 0,335633 | 0,345933 | -0,33806 | -0,32241 | 0,321667 | 0,200681 | -0,16005 | 0,160522 | 0,764787 | 0,120878 | 0,608947 | 1 | |

6.7 Appendix G Media composition for coliphage analysis

Table 19. Media composition of PBA and PTA used in double layer agar technique

| Media/Reagent | Weight | |
|---------------------------------|---------------|---------------|
| | PBA (3000 ml) | PTA (1000 ml) |
| Agar Technical | 42 g | 8 g |
| Tryptone | 39 g | 10 g |
| Glucose | 4.5 g | 3 g |
| NaCl | 24 g | 8 g |
| Na ₂ CO ₃ | 15 ml | 5 ml |
| CaCl ₂ | - | 6 ml |
| MgCl ₂ | - | 1 ml |